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Research Article

Etiological examination of neonatal calf diarrhea cases detected in Burdur region

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ABSTRACT

This work was conducted to determine the prevalence of *E. coli*, *Cryptosporidium* spp., *Giardia* sp., rotavirus and coronavirus agents involved in the etiology of diarrhea in cases of neonatal calf diarrhea detected in Burdur Mehmet Akif Ersoy University Animal Hospital Clinics and other farms in the region between 2019-2020 years. The material of the study consisted of 50 neonatal calves with diarrhea. Stool samples were taken from calves with diarrhea and examined with immunoch-romatographic rapid test kits. One or more enteropathogenic agents were detected in 70% of the calves. In calves with diarrhea, *Cryptosporidium* spp (42%) and rotavirus (30%) were detected as single and mixed enteropathogen. Mixed enteropathogens (14% *Cryptosporidium* spp. + Rotavi-rus, 2% Rotavirus + coronavirus, 2% *Cryptosporidium* spp. + rotavirus + coronavirus + *E. coli* K99 + *Giardia* spp, 2% *Cryptosporidium* spp. + coronavirus + *E. coli* K99 + *Giardia* spp) were determi-ned in 20% of calves. As a result, it was concluded that in neonatal calf diarrhea, *Cryptosporidium* spp. and rotavirus factors are common and necessary precautions should be taken in enterprises against these factors.

INTRODUCTION

The neonatal period is the most sensitive period of calf rearing, covering the first 28 days from birth. Diarrhea is one of the most important symptoms threatening the life of calves during this period. Because diarrhea increases the mortality rate in sick calves and causes great economic losses, it is very important to combat diarrhea in newborn calves (Tokgoz et al., 2013).

Diarrhea is a symptom characterized by an increase in the daily amount of stool and the number of stools, and the consistency of the stool is watery. It can be shaped due to infectious and non-infectious causes (Roy, 1980). Diarrhea in studies; Enthropathogenic bacterial species such as *Clostridium perf-ringens* (*Cl. Perfringens*), *Escherichia coli* (*E. coli*), *Salmonella* spp., *Chlamydia* spp., and *Camphylobacter jejuni*, Viruses such as Rotavirus, coronavirus, Coccidia, *Cryptosporidium* spp and *Giardia* spp, environmental factors, care and nutritional disorders, enzyme deficiencies and the immunological status of the calf have been reported to play a role (Kalınbacak, 2003; Citil, 2013).

It has been determined that the risk of diarrhea in neonatal calves varies between 15% and 20% between 0 and 30 days, and mortality rates in calves with diarrhea are between 0.2% and 8% in studies conducted in developed countries (Bendali et al, 1999). It has been reported that 75% to 90% of neonatal calf diarrhea is associated with rotavirus, coronavirus, enterotoxigenic *E. coli* and *Cryptosporidium* spp. (Senturk, 2018).

Rotavirus usually causes diarrhea in 1 to 2 week old calves. The virus has a very short incubation period of 12-24 hours and induces peracute diarrhea in affected calves. The virus replicates in the cytoplasm of small intestinal villus epithelial cells. Viral infection causes villous atrophy and usually affects the caudal part of the small intestine. After being infected, calves shed large amounts of virus in the feces for 5-7 days (Steele et al., 2004).

Coronavirus infection can occur in cattle as three separate clinical pictures. These symptoms are a diarrheal complex consisting of winter dysentery in 1-2 week old calves and hemorrhagic diarrhea in adult animals and a complex of bovine respiratory disease in both young and adult cattle (Liu L, et al., 2006). The pathogenesis of coronavirus and rotavirus is

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similar. However, atrophy of both the small and large intestines is more common in coronavirus infections (McGavin and Carlton, 1995).

The first 4 days after birth is the period when newborn calves are most susceptible to *Escherichia coli* infection (Foster and Smith, 2009). *E. coli* septicemia is common in neonatal cases. The most common cause of diarrhea in calves is entero-toxigenic *Escherichia coli* (ETEC) (Nataro and Kaper, 1998).

Infected calves with *Cryptosporidium* spp may be asymptomatic or develop severe diarrhea leading to dehydration (Fayer R., 2010). The disease causes severe villous atrophy and causes long-term malnutrition and growth retardation in infected animals (Nydam and Mohammed, 2005). Although calves can be infected with giardiasis from the age of 4 days, the 4-12 week period is the period in which stool cyst distribution is most common (O'Handley et al., 2003).

Neonatal calf diarrhea is a complex condition due to its multifactorial nature. Therefore, considering the risk factors of each pathogen that detects pathogens with laboratory tests and ensuring correct farm management may be beneficial in controlling calf diarrhea. With the rapid test kits developed in recent years, the diagnosis of some of the factors that cause diarrhea in calves can be determined quite easily under field conditions, without the need for a laboratory environment and experienced personnel.

This study was carried out to determine the prevalence of E coli, cryptosporidium spp, giardia, rotavirus and coronavirus agents in newborn calves brought to Burdur Mehmet Akif Ersoy University Animal Hospital Clinics and diagnosed with diarrhea in farms in the center and districts of Burdur between 2019-2020.

MATERIAL AND METHODS

The animal material of the study was collected from 20 Simmental, 24 Holstein, and 6 other breeds, at the age of 1-30 days, with neonatal calf diarrhea detected in Burdur Mehmet Akif Ersoy University Animal Hospital Clinics and other farmers in the region between 2019-2020. (Table 1).

Table 1. Gender distribution of calves with neonatal diarrhea.

Gender	Number of Calves (n)	Ratio to Total Number of Calves (%)
Female	21	%42
Male	29	%58
Total	50	

General clinical examinations of calves with diarrhea were performed. In stool samples, Commercial in vitro Rapid Diagnostic Test (BoviD-5 Ag Test kit, Bionote, Inc. Korea) was used for the detection of rotavirus, coronavirus, cryptosporidium, *E. coli* (K99) and *Giardia* spp. The tests were performed according to the manufacturer's test procedure.

RESULTS

In this study, while no agent could be detected in 30% (15/50) of calves with diarrhea, some enteropathogenic agents were detected in 70% (35/50) of them. Of these enteropathogenic agents, 24% (12/50) of the calves were *Cryptosporidium* spp., 12% (6/50) rotavirus, 6% (3/50) *E. coli* K99, 6% (3/50) of the calves *Giardia* spp, 2% (1/50) coronavirus, 14% (7/50) *Cryptosporidium* spp. + Rotavirus, 2% (1/50) Rotavirus + coronavirus, 2% (1/50) *Cryptosporidium* spp. + rotavirus + coronavirus + *E. coli* K99 + *Giardia* spp, *Cryptosporidium* spp. + coronavirus + *E. coli* K99 + *Giardia* spp were detected (Table 2). Mixed enteropathogen was detected in 20% (10/50) of the calves. In calves with diarrhea, *Cryptosporidium* spp. at the rate of 42% (21/50) and rotavirus at the rate of 30% (15/50) were detected as single and mixed enteropathogens.

Table 2. Distribution rates of enteropathogen agents in calves with neonatal diarrhea

Agent	Number of Animals (n=50)	Ratio (%)
Positive	**35**	**70**
Cryptosporidium spp	12	24
Rotavirus	6	12
E.Coli K99	3	6
Giardia spp	3	6
Coronavirus	1	2
Cryptosporidium spp + Rotavirus	7	14
Rotavirus+ Coronavirus	1	2
Cryptosporidium spp + Rotavirus+ Coronavirus+*E.Coli* K99+Giardia spp	1	2
Cryptosporidium spp + Coronavirus+*E.Coli* K99+Giardia **Negative**	1	2
	15	**30**

The incidence of *E. coli* as a single and mixed enteropathogen was 10%, the rate of *Giardia lamblia* as a single and mixed enteropathogen was 10%, and the rate of coronavirus as a single and mixed enteropathogen was determined as 8%. Treatment for the agent detected and symptomatic treatment protocols were applied to the calves with diarrhea. The treatment results of the calves were followed up by calling the owners, 26 calves recovered and 11 died. 13 patient owners could not be contacted. The data of the calves used in the study are given in Table 3.

When the distribution of neonatal diarrheal calves with enteropathogenic agents according to age groups is examined, 13 (37.14%) of diarrhea cases are at the age of 1-7 days, 11 (31.43%) at the age of 8-14 days, 7 (20%) at the age of 15-21 days, and at the age of 15-21 days. It was determined that 4 of them (11.43%) were 22-30 days old (Table 3). Of the causative neonatal calves with diarrhea, 17 (48.57%) were female and 18 (51.43%) were male. When the recovery rates of the calves with neonatal diarrhea were examined, the information that 7 of the calves died and 19 of them recovered was obtained from the owners of the patients, and the owners of 9 calves could not be reached. 18 of these calves were from Holstein, 14 of them were from Simmental and 3 of them were from other breeds.

DISCUSSION

Detection of enteropathogens in stool with rapid diagnostic kits has advantages compared to other diagnostic methods, such as rapid results in field conditions, inexpensive, simple and easy application, analysis of more than one factor at the same time, and determination of treatment strategies in a short time, without the need for a laboratory environment (Kaya and Coşkun, 2018).

Many researchers in our country, in various provinces, conducted studies to reveal the etiological factors in diarrhea cases of calves in the neonatal period with different methods such as agent isolation, search for parasites in the stool, serological

Table 3. Data of calves with detected enteropathogen

	Current status of the patient	Patient's race	Age (days)	Gender	Agent				
1.	Died	Holstein	20	Male	*Cryptosporidium* spp				
2.	Healed	Holstein	11	Female	*Cryptosporidium* spp	Rota virus			
3.	Healed	Norvegian red	6	Female		Rota virus	Coronavirus		
4.	Healed	Simmental	8	Female	*Cryptosporidium* spp	Rota virus			
5.	Not reached	Holstein	22	Female					*Giardia* spp
6.	Healed	Simmental	2	Male				*E.coli*	
7.	Not reached	Simmental	10	Male	*Cryptosporidium* spp	Rota virus			
8.	Healed	Hybrid	10	Male			Coronavirus		
9.	Healed	Simmental	10	Female	*Cryptosporidium* spp				
10.	Healed	Simmental	7	Male	*Cryptosporidium* spp	Rota virus			
11.	Not reached	Simmental	24	Male		Rota virus			
12.	Died	Holstein	10	Female					*Giardia* spp
13.	Died	Belgian blue	10	Female	*Cryptosporidium* spp		Coronavirus	E.*coli*	*Giardia* spp
14.	Not reached	Simmental	11	Male	*Cryptosporidium* spp				
15.	Died	Holstein	15	Female	*Cryptosporidium* spp	Rota virus	Coronavirus		*Giardia* spp
16.	Healed	Holstein	3	Female		Rota virus			
17.	Not reached	Holstein	11	Female		Rota virus			

Table 3 Continue. Data of calves with detected enteropathogen

	Current status of the patient	Patient's race	Age (days)	Gender	Agent			
18.	Not reached	Holstein	15	Male	*Cryptosporidium* spp			
19.	Healed	Simmental	3	Male		Rota virus		
20.	Healed	Holstein	10	Male	*Cryptosporidium* spp	Rota virus		
21.	Died	Holstein	4	Male	*Cryptosporidium* spp			
22.	Healed	Simmental	8	Male	*Cryptosporidium* spp			
23.	Died	Holstein	15	Female	*Cryptosporidium* spp			
24.	Not reached	Simmental	7	Male	*Cryptosporidium* spp			
25.	Not reached	Simmental	5	Female	*Cryptosporidium* spp			
26.	Healed	Holstein	15	Female	*Cryptosporidium* spp	Rota virus		
27.	Healed	Simmental	4	Male	*Cryptosporidium* spp	Rota virus		
28.	Healed	Holstein	15	Male	*Cryptosporidium* spp			
29.	Healed	Simmental	2	Female			E.*coli*	
30.	Healed	Holstein	15	Male	*Cryptosporidium* spp			
31.	Died	Simmental	28	Female				*Giardia* spp
32.	Not reached	Holstein	2	Female			*E.coli*	
33.	Healed	Holstein	7	Male	*Cryptosporidium* spp			
34.	Healed	Holstein	7	Male		Rota virus		
35.	Healed	Holstein	11	Female		Rota virus		

examinations or the use of rapid test kits (Kaya and Coşkun, 2018).

In this study, calf stool samples were taken and immunochromatographic test kits were used, which can diagnose different enteropathogens within 10-15 minutes and give rapid results (Klein et al., 2009). Mixed enteropathogens, in which at least two of these disease factors were found together, were diagnosed in 10 of 35 patients evaluated in the study. 5 enteropathogens were diagnosed together in 1 of these 10 patients, 4 enteropathogens were diagnosed together in 1, and 2 enteropathogens were diagnosed together in 8 of them. Diarrhea caused by a single enteropathogen was detected in 25 patients. Of these 25 patients, 12 of them were cryptosporidium spp., 6 of them rotavirus, 3 of them *Giardia lamblia*, 3 of them *E. coli* K99 and 1 of them coronavirus, alone caused diarrhea (Table 2). Diarrhea in mixed infections can develop as osmotic and secretory. In such cases, rotavirus, coronavirus, *Cl. Perfringens*, *Cryptosporidium* and *E. coli* two or more factors may coexist, leading to the development of osmotic and secretory diarrhea. It has been reported that mixed infections increase the severity of the disease (Baljer and Wieler, 1989).

Among the parasitic agents of neonatal diarrhea, *Cryptosporidium* spp constitutes a zoonotic disease with diarrhea, with a high probability of death in young and immune-compromised patients (Birdane, 2017). In studies conducted in our country, ((Kulig and Coskun, 2019) determined *Cryptosporidium* spp at a rate of 7% by rapid ELISA method (Citil, 2003; Al and Balikci, 2012), but they could not detect *Cryptosporidium* spp in calves with diarrhea with the same method. Emre and Fidancı (Emre and Fidanci, 1998) detected *Cryptosporidium* spp infection in feces with a rate of 63.3% using the safranin methylene blue technique. Çitil et al. (2003) found 32.9% *Cryptosporidium* spp. in stool samples in a study they conducted in Kars region. Kaya and Coşkun (2018) found

the rate of *Cryptosporidium* spp to be 11.21% in the province of Tokat. Külig and Coşkun (21) detected 7% of cryptosporidi-um spp in calves with diarrhea in Sivas. In the calves used in this study, *Cryptosporidium* spp the incidence as a single and mixed enteropathogen was found to be 42%. This rate differs from the values reported by other researchers. This difference may be due to the different methods used.

Rotavirus infections are one of the important causes of diarrhea in the neonatal period. This virus usually causes infection in calves aged 4-21 days, with 100% morbidity and 2% mortality (Sahal, et al., 2018). In studies conducted in our country in calves with neonatal diarrhea (Duman R ve Aycan AE., 2010), rotavirus infections were found between 8.5-53%. Kaya and Coşkun (Kaya and Coskun, 2018) found rotavirus infection in Tokat province to be 44.86%. Kulig and Coskun (Kulig and Coskun, 2019) found rotavirus infection at a rate of 22% in calves with diarrhea in Sivas. In studies related to the subject abroad, it has been reported that the incidence of this factor varies between 7-80% (Azkur and Aksoy, 2018). The incidence of rotavirus as a single and mixed enteropathogen in the calves used in this study was found to be 30%. This rate is between the percentiles reported by other researchers. In studies conducted in Turkey, the prevalence of rotavirus infections in calves with diarrhea at the age of 1-28 days was found to be between 0-53% (Alkan, 1998). The fact that a total of 35 calves with diarrhea included in this study were in this age range was found to be compatible with the average age range determined in previous studies.

Escherichia coli is one of the leading factors among the bacteria that cause neonatal diseases in calves. It is a factor that causes fatal diarrhea in the first week of neonatal life from one day old. It mostly causes diarrhea in calves younger than 4 days old (Sahal, et al., 2018). In studies conducted in our country, E. coli infections were found between 11.88-69.3% (Sen, et al., 2013). Kaya and Coskun (Kaya and Coskun, 2018) detected 7.48% of E. coli in Tokat province. Küliğ and Coşkun (Kulig and Coskun, 2019) determined E. coli at a rate of 26% in mixed form in calves with diarrhea in Sivas. The incidence of E. coli as a single and mixed enteropathogen in the calves used in this study was determined as 10%. This rate is lower than what many researchers have reported. This difference may be due to the different methods used.

Giardia lamblia is another parasitic agent of parasitic diarrhea in newborns. It is a protozoan that can be found in the small intestines during the 4-day to 12-week period of the neonatal period (Kaya and Coskun, 2018). In studies conducted in our country, *Giardia lamblia* infections were detected between 1.98% and 16.82% (Sen et al., 2013). Kaya and Coskun (Kaya and Coskun, 2018) found giardia to be 16.82% in Tokat pro-vince. In studies related to the subject abroad, it is stated that the incidence of this factor varies between 17-73% (Sen et al., 2013). The incidence of *Giardia lamblia* as a single and mixed enteropathogen in the calves used in this study was determi-ned as 10%. This rate is among the percentiles reported by researchers in our country.

Coronavirus is another important viral diarrhea agent in the neonatal period. It mostly causes approximately 100% morbidity and 50% mortality in 4-21-day-old calves (Şahal M, et al., 2018), (Gül Y., 2016). In studies conducted in our country, coronavirus infections were detected between 1.04% and 37.1% (Uyunmaz et al., 2019). Kaya and Coskun (Kaya and Coskun, 2018) found the rate of coronavirus in Tokat to be 9.35%. Küliğ and Coşkun (Kulig and Coskun, 2019) detected 9% mixed coronavirus infection in calves with diarrhea in Sivas. In studies related to the subject abroad, it is stated that the incidence of this factor is between 3 and 80.8% (Citil et al., 2003). The incidence of coronavirus as a single and mixed enteropathogen in the calves used in this study was determined as 8%. This rate is in line with the studies conducted.

Diarrhea factors detected in this study are found at different rates, both in our country and abroad, it can be affected by many factors such as the number of materials used, the variety of methods, the diversity of the causative population in barns or businesses, the awareness and sensitivity of animal owners to neonatal calf diarrhea, the season in which the study was conducted, and the geographical region. The results obtained in this study, when compared with other studies, Cryptosporidium spp. and E. coli rates are similar. (Baljer and Wieler 1989; Kulig and Coskun, 2019)

As a result, in neonatal calf diarrhea, *Cryptosporidium* spp and rotavirus agents are common and necessary precautions should be taken in enterprises against these agents, attention should be paid to the care and nutrition of calves against these enteropathogens as well as other enteropathogens, colostrum management for calves to receive adequate colostrum and vaccination programs for pregnant cows and calves concluded that it should be implemented.

DECLARATIONS

Ethics Approval

Burdur Mehmet Akif Ersoy University Experimental Animals Local Ethics Committee Presidency, E-93773921-770-160273, Etiological examination of neonatal calf diarrhea cases detected in Burdur region, due to the use of fecal material, Animal Experiments Ethics Committees dated 15 February 2014 and numbered 28914. According to article 8 of the Regulation on Working Procedures and Principles, Ethics Committee Approval is not required.

Conflict o Interest

There is no conflict of interest.

Consent for Publication

N/A

Author contribution

Idea, concept and design: HAA, MK

Data collection and analysis: RK, SS, YM

Drafting of the manuscript: RY, HIG, NM, SSD

Critical review: HAA, MK

Data Availability

The data is available from the corresponding author on reasonable request.

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REFERENCES

1. Alkan, F. (1998). The role of rotavirus and coronaviruses in calf diarrhea. Journal of Ankara University Faculty of Veterinary Medicine. 45, 29-37.

2. Al, M., & Balikci, E. (2012). Diagnosis of rotavirus, coronavirus, *E. coli* K99 and *Cryptosporidium parvum* with rapid test kits in calves with neonatal diarrhea and the relationship between enteropathogen and maternal immunity. Fırat University Journal of Health Sciences Medicine. 26(2):73-78.

3. Azkur, A.K., & Aksoy E. (2018). Preventive measures in calf diseases. Lalahan Animal Res. Inst. Journal. 58(Special issue): p.56-63.

4. Baljer, G., & Wieler, L.Ä. (1989). Pathogenese und immunprophylaxe der neonatalen Durchfallerkrankungen der Kälber. Vet. 5: 18-26.

5. Bendali, F., Bichet, H., Schelcher, F., & Sanaa, M. (1999). Pattern of diarrhea in newborn beef calves in south-west France. Veterinary Research 30, 61–74.

6. Birdane, M.F. (2017). Cryptosporidiosis diarrheas in farm animals. Kocatepe Veterinary Journal.10(2): 91-98.

7. Citil, M., Gunes, V., Karademir, B. (2003). A retrospective study on diarrheal calves brought to KAU veterinary faculty internal medicine clinic between 1996-2001. Vet. Fac. Journal. 39-42.

8. Duman, R., & Aycan, A.E. (2010). Prevalence of rotavirus infection in calves with diarrhea in Konya region. Journal of Anim and Vet. Advances. 9(1):136-138.

9. Emre, Z. & Fidancı, H. (1998). Prevalence of mix infections of *Cryptosporidium* spp., *Escherichia coli* K99 and rotavirus in the faeces of diarrheic and healthy cattle in Ankara, Turkey and in vitro resistance of Escherichia coli K99 to antimicrobial agents. Tr. J. Of veterinary and Animal Science. 22:175-178.

10. Fayer R. (2010). Taxonomy and species delimitation in *Cryptosporidium*. Exp. Parasitol. 124:90–97.

11. Foster, D.M., & Smith, G.W. (2009). Pathophysiology of diarrhea in calves. Vet Clin North Am Food Anim Pract. 25:13–36.

12. Gul, Y. (2016). Internal diseases of ruminant animals. Medipres Publishing. 4th Edition.

13. Kalınbacak, A. (2003). The use of hypertonic salindextran and oral electrolyte solution in fluid therapy of calves with diarrhea. AU Vet Fak Journal. 50, 113-118.

14. Kaya, U., & Coskun, A. (2018). Determination of the etiology of neonatal calf diarrhea in the Tokat region. Manas J Agr Vet Life Sci.; 8 (1), 75- 80.

15. Klein, D., Kern, A., Lapan, G., Benetka, V., Möstl, K., Hassl, A., & Baumgartner, W. (2009). Evaluation of rapid assays for the detection of bovine coronavirus, rotavirus A and *Cryptosporidium parvum* in faecal samples of calves. Vet J.182: 484–486.

16. Kulig, C.C., & Coskun, A. (2019). Prevalence of *E. coli*, *Cryptosporidium*, *Clostridium perfringens*, rotavirus and coronavirus in neonatal diarrheal calves in Sivas and its districts. Turk Vet. J.1(2):69-73

17. Liu, L., Hägglund, S., Hakhverdyan, M., Alenius, S., Larsen, L.E., & Belák, S. (2006). Molecular epidemiology of bovine coronavirus on the basis of comparative analyses of the S gene. J Clin Microbiol. 44:957–960.

18. McGavin, M.D., & Carlton, W.T. (1995): Special Veterinary Pathology, 2nd ed, St. Louis: Mosby. p. 48-58.

19. Nataro, J. P., & Kaper, J.B. (1998). Diarrheagenic Escherichia coli, Clinical Microbiology Reviews. Vol 11, No.1. p. 142-201.

20. Nydam, D.V., & Mohammed, H.O. (2005). Quantitative risk assessment of *Cryptosporidium* species infection in dairy calves. J Dairy Sci. 88: 3932–3943.

21. O'Handley, R.M., Ceri, H., Anette, C., & Olson, M.E. (2003). Passive immunity and serological immune response in dairy calves associated with natural *Giardia duodenalis* infections. Vet Parasitol;113(2): 89-98.

22. Roy, J.H.B. (1980). Factors affecting susceptibility of calves to disease. J Dairy Sci, 63(4), 650-664.

23. Steele, A.D., Geyer, A., & Gerdes, G.H. (2004). Rotavirus infections. In: Coetzer JAW, Tustin RC, editors. Infectious Diseases of Livestock. 2nd ed. Cape Town: Oxford University Press Southern Africa; p. 1256–1264.

24. Sahal, M., Terzi, O.S., Ceylan, E., & Kara, E. (2018). Calf diarrhea and prevention methods. Lalahan Hay. Research. Ins. Journal. 58 (special issue): p. 41-49.

25. Sen, I., Güzelbektas, H., & Yıldız, R. (2013). Neonatal Calf Diarrhea: Pathophysiology, Epidemiology, Clinic, Treatment and Prevention.Turkiye Klinikleri J Vet Sci. 4(1), 71-78.

26. Sentürk, S. (2018) Internal Diseases of Calves with Case Controversial. F Özsan Matbaacılık San ve Tic Ltd Şti, Bursa., Extended 3. Edition.

27. Tokgoz, B.S., Ozdemir, R., Turut, N., Mirioglu, M., Ince, H., Mahanoglu, B., Yoldas, A., & Tuzcu, N. (2013). Determination of morbidity and mortality and risk factors of neonatal calf infections in Adana region. AVKAE Journal. 3(1):7-14.

28. Uyunmaz, S.G., Bulut, O., Hasoksuz, M., & Hadimli, H.H. (2019). Investigation of bovine coronavirus and bovine rotavirus by rapid diagnosis kit and RT-PCR in diarrheic calf feces. Journal of Istanbul Veterinary Science. 3(3):57-63.

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Research Article

Pathomorphological examination of hydatid cyst in lungs and livers of cattles slaughtered in a commerical abattoir in Eskişehir

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ABSTRACT

Hydatid cyst or Cystic Echinococcosis continues to be an important problem in terms of public health as well as animal health in regions with low socio-cultural level where preventive medicine cannot be fully performed. In this study, it is aimed to make an evaluation about the situation of the cattle slaughtered in the region by examining the lungs and livers of the cattle slaughtered in a private abottoir in Eskisehir in terms of hydatid cyst. For this purpose, 150 cattle cuts were observed and tissue samples were taken from 36 lungs and 21 livers of 46 animals due to suspicious cyst structure in macroscopic examinations. They were fixed in 10% buffered formalin solution for histopathology. After routine histopathology process, 5 micron sections were taken from paraffin blocks. Each section was stained according to the procedure with HE, MT and PAS stains, respectively. At the end of the research, hydatid cyst was determined in 26 of 150 cattle (17.33 %). At the organ level; The numbers of hydatid cyst seen in the lung is 22 (14.67%), in the liver is 10 (6.67%) and 6 (4%) both in the lung and liver. In microscopic examinations, protoscolex was also observed in 18.18% of hydatid cysts located in the lung and 20% of cysts located in the liver. As a result, 17.33% hydatid cyst was determined in the cattle slaughtered in slaughterhouse in Eskisehir. It is concluded the disease should be considered for public health in this region in terms of its zoonotic character.

INTRODUCTION

Hydatid cyst (HC) or Cystic Echinococcosis (CE) is a zoonotic, parasitic infection that manifests itself with fluid-filled cysts in intermediate host organs by the larvae of the parasite *Echinococcus granulosus*. It is more common in places where pasture farming is common and in areas where illegal animal slaughter cannot be prevented (Sayır and Çobanoğlu, 2013, Tilkan et al., 2018). The genus Echinococcus is a parasite belonging to the Taenidea family of the Cyclophylidea order from the Cestoda subclass of the Cestoidea class, and 4 species are known today. These; *Echinococcus granulosus, Echinococcus alveolaris (multiocularis), Echinococcus vogali* and *Echinococcus oligarthus*. The most common species in the world are; *Echinococcus granulosus* and *Echinococcus alveolaris* (Eckert and Deplazes, 2004; Tilkan et al., 2018). *Echinococcus granulosus* has 10 genotypes (G1-G10) identified so far. Of these 10 different genotypes, G1 and G2 were detected in sheep, G3 and G5 cattle, G4 horses, G6 camels, G7 and G9 pigs, G8 deer and G10 reindeer (Thompson and Lymbery, 1995; Thompson and McManus, 2002; McManus and Thompson, 2003). It is stated that at least 7 of these strains are contagious to humans (Eckert and Deplazses, 2004).

The adult form of *Echinococcus granulosus* settles in the intestines of dogs, foxes, wolves and coyotes. The larval form of the parasite is intermediate in humans and herbivores; It is known as Hydatidosis, Cystic Echinococcosis or Hydatid Cyst (Fisser, 1988; Craig and Regan, 2003; Sayır and Çobanoğlu, 2013). The disease is transmitted by the fecal-oral route. Infected eggs produced by the parasite living in the small intestine of the main host are excreted with the feces. Intermediate host animals and humans ingest these eggs. Oral oncospheres are released by the action of pepsin enzyme in the stomach and bile salts in the duodenum. The oncospheres released in the duedonum are attached to the intestinal wall with their hooks. Oncospheres that enter a small vein or lymph duct with their destruction of the intestinal wall are passively transported to the liver. While most of the oncospheres are attached to the liver, the ones that cannot be adhered to come to the right ventricle of the heart and are carried from there to the lungs, where the oncospheres are most frequently attached. Apart from these, oncospheres can also go to all tissues and organs of the body via the aorta and settle in different tissues (McManus et al 2003; Yıldız and Tunçer, 2005; Tilkan et al., 2018; Wen et al., 2019). About 5 days after ovulation, the metacestode consists of an inner cellular layer (germinal layer) and an outer acellular (laminar) layer with a diameter of 60-70 µm. The cyst enlarges and turns into a granulomatous

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tissue with the host reaction after a while. Afterwards, a typical appearance is formed by the fibrous tissue reaction. Although the exact time required for the development of protoscolex cannot be defined, it is reported that it may take more than 10 months after infection (Eckert and Deplazses, 2004).

Hydatid cysts are uni or multilocular cysts lined with germinal epithelium and consist of three layers. These are from the inside out; They are defined as endocyst, exocyst and pericyst. Endocyst is the germinal layer where budding takes place. The exocyst (cuticle) forms the outer layer of the cyst. It has selective permeability. The pericyst is not a parasitic structure but a fibrous capsule formed by the host (Beigh et al., 2018). Typical layered structure of hydatid cysts, necrosis around the cyst wall, concomitant inflammatory cell reaction, and formation of foreign body giant cells are the defining findings in HE staining of histopathological sections (Beigh et al., 2017a; Beigh et al.; 2017b). It is reported that PAS staining of the acellular laminar layer of the cyst will be confirmatory in the histopathological diagnosis of the disease (OIE, 2017).

Different results have been seen in slaughterhouse studies on HC in Turkey. Öge et al. (1998) examined the liver and lungs of 1941 cattle slaughtered in their slaughterhouse study in Ankara and found HC in 185 animals (9.5%). Researchers reported 32.9% involvement in the lung, 30.2% in the liver, and 35.6% in both organs together. In the study conducted by Celep et al. (1990) in Samsun region, the rate of HC in cattle was reported as 21.1%. In the study conducted by Gıcık et al. (2004) in Kars, HC was reported in 150 (31.25%) of 480 cattle, and the rate of localization in the liver was 80%, the rate of localization in the lung was 64%, and the involvement in both organs was 44%. Researchers reported the rate of cystic echinococcosis in sheep as 63.85% in the same study. In a study conducted in Kırıkkale, HC was found in 120 (14.17%) of 847 cattle brought to the slaughterhouse. It has been reported that 49.16% of positive cases are located in the lung, 16.68% in the liver, and 34.16% in both the lung and liver (Yıldız and Tunçer, 2005). In another study conducted in Sivas, the lungs and livers of 765 cattle slaughtered were examined and HC was found in 273 animals (35.7%). These cysts were seen in the lungs of 103 cattle (13.5%), in the liver of 66 cattle (8.63%), and they were reported together in the lungs and liver (13.6%) of 104 cattle (Acıöz et al., 2008).

In a study conducted in cattle slaughtered in 3 different slaughterhouses in Afyonkarahisar, CE was found with a rate of 29.47%, the detected cysts were reported to be in only the liver with a rate of 44.06%, and only in the lung with a rate of 30.85% and in the liver and lung at a rate of 23.73% (Köse et al., 2008). In another study conducted in Kayseri, HC was found in the livers of 9 (3%) of 300 cattle slaughtered in a slaughterhouse (Düzlü et al., 2010). In a study involving Ordu and Erzurum regions, Fidan and Kapakin Terim (2016) detected 12% HC as a result of macroscopic and microscopic examination of the lungs of the cattle slaughtered in the slaughterhouse. On the provincial basis, the researchers reported the incidence of hydatid cysts in the lungs of cattle as 14.86% in the Erzurum region and 11.26% in the Ordu region.

It is seen that HC is an important zoonotic disease that affects animal and human health together. No study was found regarding the presence of HC in the cattle slaughtered in Eskisehir in the literature surveys made as of the time of the study. In this study, it was aimed to examine the pathomorphological HC status of slaughtered cattle in Eskisehir, which is among the leading cities in the agriculture and livestock sector in the Central Anatolia Region.

MATERIAL and METHODS

Study Material

After obtaining permission from a private slaughterhouse operating in Eskisehir, 150 cattle cuts were examined. Belonging to 46 animals that were suspicious in macroscopic examinations; a total of 57 samples (36 lungs and 21 liver) were taken. After the samples were numbered, they were placed in 10% buffered formaldehyde solution and brought to the Laboratory of the Department of Pathology, Faculty of Veterinary Medicine, University of Selcuk for histopathological examinations.

Histopathology Procedures and Evaluation

The samples, which were reduced appropriately for paraffin blocking, were taken into numbered tissue cassettes and kept for 10-12 hours in a freshly prepared 10% buffered formalin solution for the second time. After fixation, they were kept under running water for 12 hours in order to remove the formalin effect from the tissues. After washing, the tissues were transferred to an automatic tissue tracking device (Leica TP 1020) for dehydration and xylol processes. Graded alcohol-xylol treatments were completed in accordance with the device instructions and tissue follow-up protocol (Luna, 1968). Paraffin blocks were prepared from tissue samples whose dehydration and transparency processes were completed. By means of a microtome (Leica RM 2125 RT), sufficient 5 micron sections were taken for HE, PAS and MT staining and kept in an oven at 37 0C until staining. All sections were first stained with HE (Hematoxylin-Eosin) and the needed ones were stained with MT (Masson's Trichrome) and PAS (Periodic Acid Schiff) staining methods (Luna, 1968; Presnell and Schreibma, 1997). During the microscopic examinations, the photographs of the suitable samples were taken (Olympus DP12 Microscopic Digital Camera Systems).

RESULTS

Macroscopic Findings

In macroscopic examinations; It was observed that the cysts observed in the lung and liver were generally protruding from the organ surface, and their free parts were surrounded by a hard and mostly transparent cyst wall. In the measurements made, it was determined that the cysts ranged between 0.2-6 cm (Figure 1). The macroscopic location information of hydatid cysts in the lung and liver is given in Table 1.

Microscopic Findings

HC structures in 4X, 10X and 20X examinations of HE

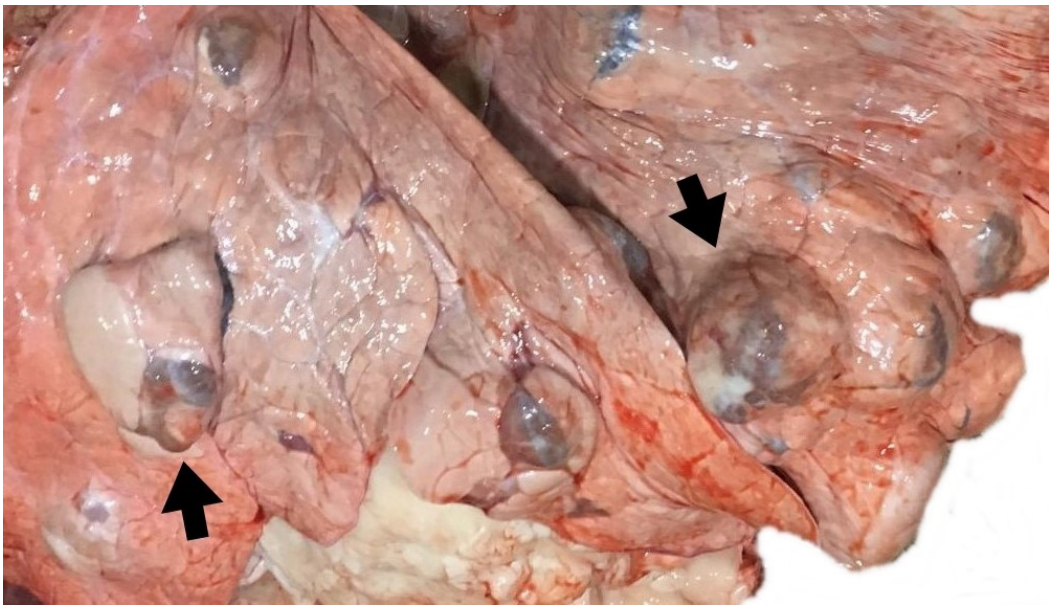

Figure 1. Multiple and different sized HC structures. Lung

Table 1. Macroscopic location of hydatid cysts in the lung

Organ	Type of the cysts		Location of the cysts		
Lung	Single cyst	Multiple cysts	Cranial	Caudal	Both sides
	12	10	7	11	4
Liver	Single cyst	Multiple cysts	Dorsal	Ventral	Both sides
	8	2	4	5	1

stainings, respectively; the innermost slightly pink colored germinal layer was surrounded by a more pink homogeneously colored outer laminar layer (cuticle) located adjacent to the germinal layer from the outside. The parasite had a typical granuloma appearance with necrosis around the cyst wall, an area of inflammatory cells of varying density, and fibrous encapsulation at the outermost part (Figure 2, a and b). In larger objective (X40) examinations of histopathological sections, budding surface on the thin structure of the germinal layer and protoscolex formations in some sections were noted. Necrotic parenchyma tissue was observed in the area immediately adjacent to the laminar layer with a more homogeneously stained acellular laminar layer (Figure 2, c and d).

In microscopic examinations, protoscolex was detected in 4 (18.18%) of 22 lung sections and 2 (20%) of 10 liver sections. While protoscolices were mostly adjacent to the germinal layer, they were seen free within the cyst in fewer cases (Figure 3a). In most of the cases, it was observed that the inflammatory cell line surrounding the parasitic structure was surrounded by mononuclear cells, eosinophil granulocytes, epithelioid cells and foreign body giant cells, and the necrotic area sometimes calcified (Figure 3. b, c and d). It was noted that the laminar layer was prominently stained in MT staining (Figure 4, a and b). A strong fibrous proliferation was also observed around the hydatid cysts in MT staining. In PAS staining samples, it was revealed that the acellular structures of the parasitic cyst were positively stained (Figure 4, c and d).

The cases with germinal and laminar layers and/or protoscolex in microscopic examinations after HE, MT and PAS staining were evaluated as positive for HC. As a result of the study; HC was determined in 26 of a total of 150 cattle (17.33%). At the organ level, HC was detected together in 22 (14.67%) lungs, 10 (6.67%) livers, and 6 (4%) of them in both lung and liver.

In the histopathological examinations of other tissue samples that were macroscopically suspicious during the slaughterhouse examinations, but HC was not detected in the microscopic examinations; Of 20 lung samples, 7 had emphysema (6 of emphysema with cyst or pneumonia, 1 without other lesion), 3 had abscess, and 10 had pneumonia. In liver samples, abscess in 7 cases and fat degeneration in 4 cases were detected.

DISCUSSION

In a retrospective study conducted at Eskisehir Osmangazi University Medical Faculty Hospital for public health awareness purposes, serum samples of 892 patients sent to the Microbiology Laboratory with the preliminary diagnosis of CE from various clinics between 2009 and 2019 were analyzed with the *Echinococcus granulosus* IgG ELISA test. Seropositivity of 30% has been reported. In the study, it was emphasized that hospital records represent some of the cases and that CE is still a disease that maintains its importance in Eskisehir (Doğan et al., 2020). In the literature review of CE disease, which is seen as a zoonosis of great importance in terms of public health, no pathology-oriented study was found that revealed the presence of HC in cattle slaughtered in Eskisehir as of the period of the thesis study. In 150 cattle included in

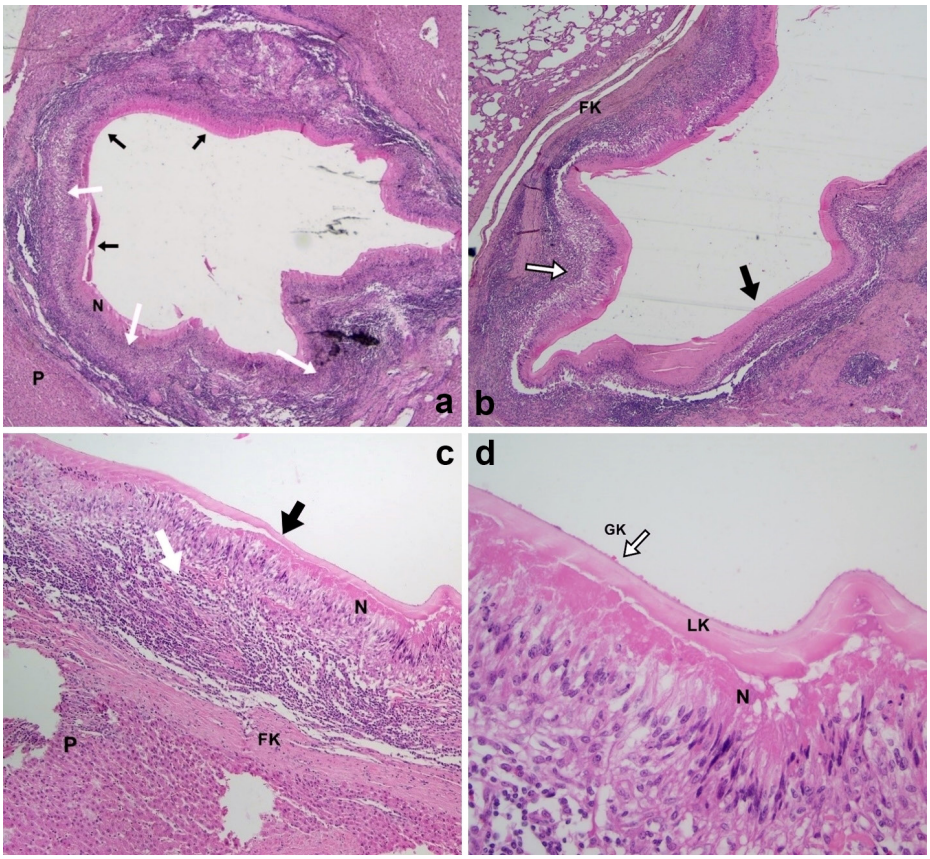

Figure 2. General view of CE and environmental structures. **a)** germinal and laminar layer (black arrows), area of necrosis (N), inflammatory infiltration (white arrow) and liver parenchyma (P), liver, HE, 4X **b)** germinal and laminar layer (black arrow), inflammatory infiltration zone (white arrow) and fibrous capsule (FK) lung, HE, 10X **c)** germinal and laminar layer (black arrow), area of necrosis (N), inflammatory infiltration (white arrow), fibrous capsule (FC) and liver parenchyma (P), liver, HE, 20X **d)** germinal layer (white arrow) laminar layer (LK), necrosis area (N) and mononuclear cell reactions with epithelioid cells further down. Liver, H-E, 40X

our study, HC was determined at a rate of 17.33% after histopathological examinations.

In the other studies on hydatid cyst in cattle in Turkey; In Samsun 21.1% (Celep et al., 1990), 3% in Kayseri (Düzlü et al., 2010), 9.5% in Ankara (Öge et al., 1998), 14.17% in Kırıkkale (Yıldız and Tunçer, 2005), 29.47% in Afyonkarahisar (Köse et al., 2008), 31.25% in Kars (Gıcık et al., 2004) and 35.7% in Sivas (Acıöz et al., 2008) have been reported. It is reported that hydatid cysts, which can be seen in every region of Turkey, are mostly encountered in Eastern Anatolia, then Central Anatolia and Marmara regions, respectively (Çobanoğlu, 2016).

Considering the above data, in our study on HC in the lungs and livers of cattle in the Eskisehir region, a lower level of cysts was observed compared to the Eastern Anatolia Region. Epidemiologically, CE in humans occurs mostly in rural areas where dogs are kept for herd protection (McManus et al., 2003). In this respect, there is an important relationship between the primary host of the parasite, carnivores, and intermediate host herbivores and humans. Turkey is defined as an endemic region in terms of CE and alveolar echinococcosis in humans (Altıntaş, 2003).

In the macroscopic examinations of the presented study, HC focus was found to be single in 12 lungs, more than once in 10 lungs, and in the liver single in 8 patients and more than once in 2 patients. While the cysts were sometimes completely lost in the parenchyma tissue, they were mostly visible from the surface. In the lung, cysts were found only in the caudal lobes in 11 cases, and only in the cranial lobes in 7 cases. In 4 cases, localization was observed in both lung regions. In the liver, it was located dorsal in 4 cases, ventral in 5 cases, and was distributed on both sides in 1 case. As Beigh et al. (2018) stated, it was thought that the looser spongy structure of the lung tissue and the wider vascular network caused the parasite larvae to spread more easily in this tissue.

After the parasite's egg is ingested by the intermediate hosts, the embryo, which is released from the egg, which is broken down by the effect of bile salts in the duodenum, passes through the jejunum and ileum wall to the portal vein, peridoudenal and perigastric lymphatics and reaches the liver first. For this reason, it has been reported that the liver is affected first at a rate of 60-70%, and the lungs are the location of the agent at a rate of 20-25%, except for the liver (McManus et al., 2003, Tilkan et al., 2018, Wen et al., 2019). In the study conducted by Gıcık et al., (2004) in Kars, it was stated that the rate of localization of cysts in the liver was 80%, and the rate of localization in the lung was 64%. Köse et al. (2008) reported that it was seen only in the liver with a rate of 44.06% and only in

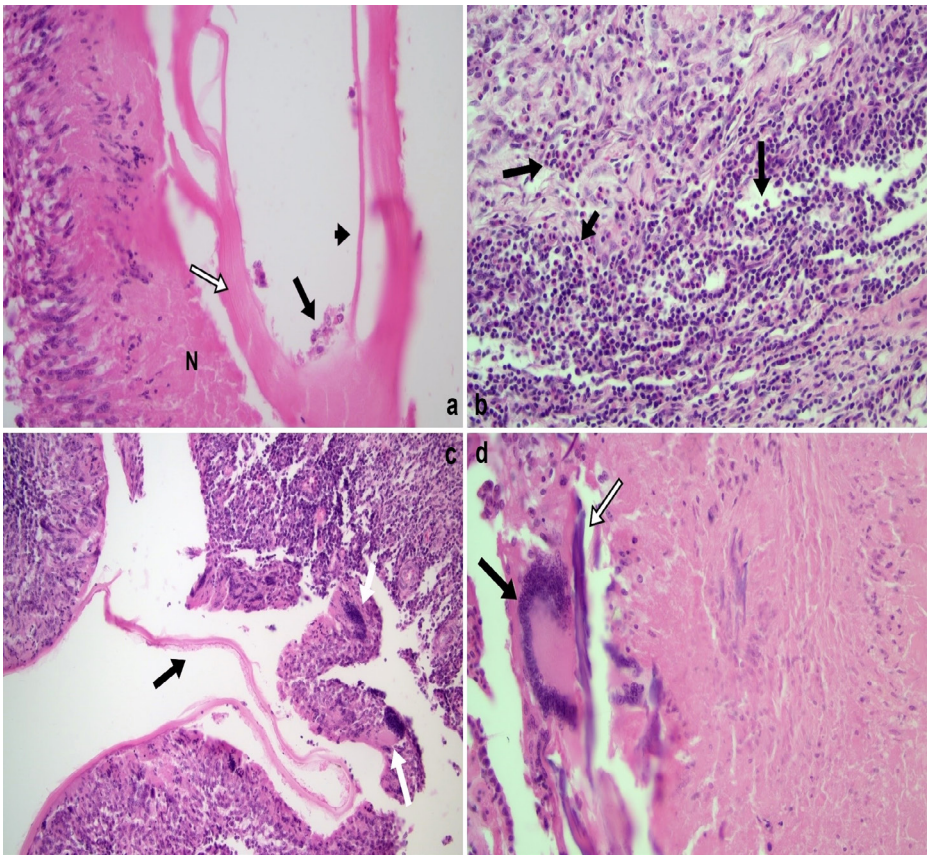

Figure 3. a) Germinal layer (black short arrow), protoscolex (black long arrow), laminar layer (white arrow), and area of necrosis (N). Lung, H-E, 40X **b)** Deposition of eosinophil granulocytes and mononuclear cells around HC (white arrow), germinal and laminar layer (black arrow), necrosis (N), H-E, 20X **c)** germinal and laminar layer (black arrow), foreign body giant cells. Lung, H-E, 10X **d)** foreign body giant cell (black arrow) and calcification (white arrow). Lung, H-E, 40X.

the lung with a rate of 30.85%. Contrary to the reported rates of HC at the organ level in our study, it was detected more in the lung with a rate of 14.67%, followed by the liver with a rate of 6.67%. It is known that the larvae usually come to the lung from the hepatic sinusoids. Apart from this, it has been reported that it can come directly through the lymphatic system or through the bronchiole channel, and in this way, the lung can be the primary localization organ by inhaling the eggs (Morar and Feldman, 2003). In our study, the rate of co-occurrence in both organs was found to be 4%. In studies where more HC was detected in the lung; Öge et al (1988) reported 32.9% in the lung, 30.2% in the liver, Yıldız and Tunçer (2005) reported 49.16% in the lung, 16.68% in the liver, Acıöz et al. (2008) reported it as 13.5% in the lung and 8.63% in the liver. Fidan and Kapakin Terim (2016), on the other hand, determined the incidence of hydatid cysts in the lungs of cattle slaughtered in the slaughterhouse to be 14.86% in Erzurum and 11.26% in Ordu, in their study involving Ordu and Erzurum regions.

In our study, HC structures in HE staining were determined as the innermost cyst as stated in the literature (Li et al., 2008; Altun and Sağlam, 2014; Fidan and Kapakin Terim, 2016; Beigh et al. 2017a; Beigh et al. 2017b; Beigh et al. 2018). The slightly pink colored germinal layer surrounding the cavity was seen as budding in places on the inner surface of this layer and protoscolex structures developed from here in some sections. In our present study, protoscolex was detected in 4 (18.18%) of 22 lung sections and 2 (20%) of 10 liver sections. Cysts are called fertile or infertile (sterile) according to the presence of protoscolex. Although the mechanisms explaining the cyst fertility status are not clear, it is stated that the host immune reaction plays a very important role in this formation. In fertile cysts, protoscolices may be tightly attached to the germinal layer or may be found freely in the cyst fluid. There is no such protoscolex structure in infertile cysts (Hidalgo et al., 2019).

The germinal layer is accepted as the parasite itself, and it is stated that the laminar layer, which supports it and forms the most resistant part of the cyst, is composed of a protein-polysaccharide complex predominantly consisting of glucosamine and galactosamine. The pericyst region, which is initiated in the early developmental stages of the post-oncospheres, is an indicator of the host's inflammatory response. The intensity of the reaction is variable for each host and also affects larval development (Solcan et al 2010). Hydatid cysts can cause advanced damage to the tissues they are located. With the disruption of the integrity of the cyst, it may spread to other organs, and may occasionally lead to anaphylactic reactions (Barnes et al., 2011). In the samples examined in our study, necrosis was observed in the regions adjacent to the laminar layer, emphysema in the alveoli in the lung, degeneration in the hepatocytes in the liver was observed around the fibrous capsule.

In the present study, a strong reaction was noted in which

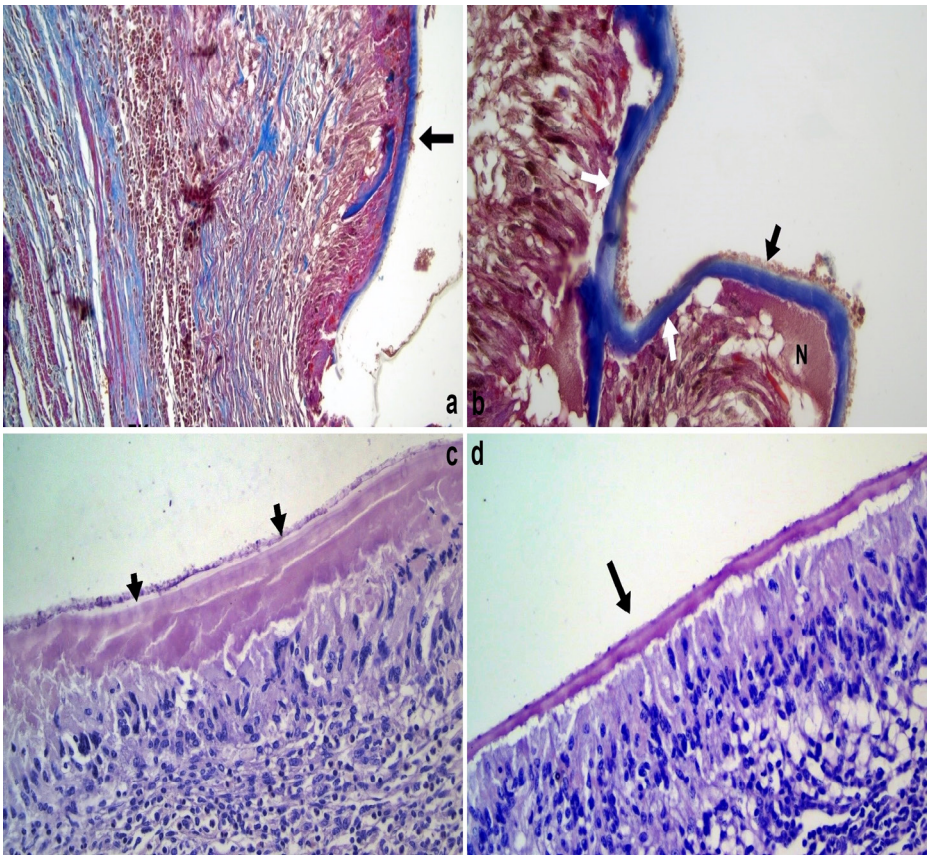

Figure 4. a) Fibrous proliferation on the left and germinal and laminar layer on the far right (black arrow), MT staining, 10X **b)** Germinal layer (black arrow) laminar layer (white arrow) and areas of necrosis (N), MT staining 40X **c)** Granular of HK and PAS (+) staining of laminar layers (black arrow), 40X, lung **d)** PAS (+) staining of granular and laminar layers (black arrows), 40X, liver.

the cysts were surrounded by a very intense fibrous proliferation. In a study on the increase in fibrous proliferation in the liver of CE patients, it was shown that miR-19 expression was significantly decreased compared to the normal liver, and it was stated that *Echinococcus granulosus* could inhibit miR-19 liver expression and lead to fibrous proliferation with a number of mechanisms (Zhang et al., 2016).

In our study, it was observed that the acellular structures of the parasitic cyst gave a positive reaction in the PAS staining samples. The acellular laminar layer is a carbohydrate protein complex containing galactose, galactosamine and glucosamine as the main component of the polysaccharide part and gives a strong response to the Schiff reaction in PAS staining. It is known that the laminar layer consists of various mucopolysaccharide and keratin structures and functions to maintain the physical integrity of the hydatid cyst and protect the germinal layer cells from host immunity. Especially in the 14-18th day of cyst formation. While it was not yet present until today, it begins to appear as a thin, transparent layer in the next process (Diaz et al., 2011; Beigh et al., 2017b). In our study, it was also noted that the laminar layer was stained quite well in MT staining. Similarly, fibrous proliferation around the HC was confirmed by MT staining.

CONCLUSION

In conclusion of the presented study, HK was determined at a rate of 17.33% after histopathological examinations in 150 cattle slaughtered in a private enterprise operating in Eskisehir. The incidence of cysts in the lung was 14.67%, the incidence in the liver was 6.67%, and the incidence of both was 4%. In microscopic examinations, protoscolex was also observed in 18.18% of hydatid cysts located in the lung and 20% of the cysts located in the liver. In line with the findings obtained in the study, it has been evaluated that HC are at a rate that should be taken into account in cattle slaughtered in the Eskisehir region, and that it may cause important problems in terms of public health as well as animal health.

DECLARATIONS

Ethics Approval

This research was approved by the decision of the Ethics Committee of Selcuk University Faculty of Veterinary Medicine, Experimental Animal Production and Research Center, dated 31.01.2019 and numbered 2019/09.

Conflict o Interest

The authors have no conflicts of interest.

Consent for Publication

Not applicable.

Author contribution

Idea, concept and design: EO, AÇE

Data collection and analysis: AÇE, EO

Drafting of the manuscript: AÇE, EO

Critical review: EO

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

1. Acıöz, M., Çeliksöz, A., Özçelik, S., & Değerli, S. (2008). Sivas'ta Nisan-Mayıs 2005 tarihleri arasında kesilen sığırlarda kist hidatik yaygınlığı. Türkiye Parazitoloji Dergisi, 32, 205 – 207.

2. Altıntas, N. (2003). Past to present: Echinococcosis in Turkey. Acta Tropica, 85, 105-12.

3. Altun, S., & Sağlam, Y.S. (2014). Erzurum ilinde kesimi yapılan sığırlarda karaciğer lezyonları üzerinde patolojik incelemeler. Atatürk Üniversitesi Veteriner Bilimleri Dergisi, 9, 7-15.

4. Barnes, T.S., Hinds, L.A., Jenkins, D.J., Bielefeldt-Ohmann, H., Lightowlers, M.W., & Coleman, G.T. (2011). Comparative pathology of pulmonary hydatid cysts in macropods and sheep. Journal of Comperative Pathology, 144, 113-122.

5. Beigh, A.B., Darzi, M.M., Bashir, S., Kashani, B., Shah, A., & Shah, SA. (2017a). Gross and histopathological alterations associated with cystic echinococcosis in small ruminants. Journal of Parasit Disease, 41, 1028-1033.

6. Beigh, A.B., Darzi, M.M., Bashir, S., Kashani, B., Shah, A., & Shah, SA. (2017b). Pathological and histochemical studies of the effects of Cystic Echinococcosis in sheep. Comparative Clinical Pathology, 22, 1-8.

7. Beigh, A.B., Darzi, M.M., Bashir, S., Shah, A., & Shah, S.A. (2018). The pathology of cystic echinococcosis and structural details of hydatid cyst and protoscolex. Indian Journal of Veterinary Pathology, 42(1), 8-14.

8. Celep, A., Açıcı, M., Çetindağ, M., Coşkun, S.Z., & Gürsoy, S. (1990). Helminthological studies on cattle from the Samsun region. Etlik Veteriner Mikrobiyoloji Dergisi, 6, 117-130.

9. Craig, P.S., Rogan, M.T., Campos Ponce, M. (2003) Echinococcosis: disease, detection and transmission. Parasitology, 127, 5-20.

10. Çobanoğlu, U. (2016) Tarihçe ve epidemiyoloji. In: İ. Yalçınkaya (ed.). Akciğer Hidatik Kisti (pp.12-19). TÜSAD Eğitim Kitapları Serisi.

11. Díaz, A., Casaravilla, C., Irigoín, F., Lin, G., Previato, J.O., & Ferreira, F. (2011). Understanding the laminated layer of larval Echinococcus I: structure. Trends in Parasitology, 27, 204-213.

12. Doğan, N., İlhan, D., & Alkan, A.Ş. (2020). ESOGÜ tıp fakültesi hastanesi mikrobiyoloji laboratuvarına gönderilen kist hidatik ön tanılı hasta örneklerinin; yaş, cinsiyet ve gönderilen bölümlere göre on yıllık laboratuvar sonuçlarının gözden geçirilmesi. Türk Tıp Öğrencileri Araştırma Dergisi, 2(3), 119-125.

13. Düzlü., Ö, Yıldırım, A., Sarıözkan, S., & İnci, A. (2010). Kayseri yöresinde üç farklı mezbahada kesilen koyun ve sığırlarda Kistik Echinococosis'in ekonomik önemi. Erciyes Üniversitesi Veteriner Fakültesi Dergisi, 7, 7-11.

14. Eckert, J., & Deplazes, P. (2004). Biological, epidemyological and clinical aspects of echinococcosis, a zoonozis of increasing concern. Clinical Microbiology Reviews, 17, 107-135.

15. Fidan, N., & Kapakin Terim, A.K. (2016). Ordu ve Erzurum yörelerinde sığır akciğerlerinin paraziter enfeksiyonlarının histopatolojik yönden incelenmesi, Atatürk Üniversitesi Veteriner Bilimleri Dergisi, 11, 40-6.

16. Fisser, A. (1988) Larval Cestodes. In: L. Collier, A. Balows, M. Susman (eds.). Topley and Wilsons Microbiology and Microbial Infections. 9th ed, (pp. 539-560), Philadelphia.

17. Gıcık, Y., Arslan, M.Ö., Kara, M., & Köse, M. (2004). Kars ilinde kesilen sığır ve koyunlarda kistik ekinokokkozisin yaygınlığı. Türkiye Parazitoloji Dergisi, 28, 136-9.

18. Hidalgo, C., Stoore, C., Strull, K., Franco, C., Corrêa, F., Jiménez, M. & Paredes, R. (2019). New insights of the local immune response against both fertile and infertile hydatid cysts. PLoS One, 14 (1), e0211542.

19. Köse, M., & Kırcalı, S.F. (2008). Prevalence of cystic echinococcosis in slaughtered cattle in Afyonkarahisar. Türkiye Parazitoloji Dergisi, 32, 27-30.

20. Li, T., Ito, A., Nakaya, K., Qiu, J., Nakao, M., Zhen, R. & Craig, P.S. (2008). Species identification of human echinococcosis using histopathology and genotyping in northwestern China. Transactions of The Royal Society of Tropical Medicine and Hygiene, 102, 585-590.

21. Luna, L.G. (1968). Manual of histologic staining methods of the armed forces institue of pathology. Mc Graw-Hill Book Company, New York.

22. McManus, D.P., & Thompson, R.C. (2003). Molekuler epidemiology of cystic echinococcosis. Parasitology. 127, 37-51.

23. McManus, D.P., Zhang, W., Li, J. & Bartley, P.B. (2003). Echinococcosis. The Lancet, 362, 1295-304.

24. Morar, R. & Feldman, C. (2003). Pulmonary echinococcosis. European Respiratory Journal, 21, 1069-1077.

25. OIE. (2017). Echinococcosis infection with E. granulosus and E. multilocularis. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.06_ECHINOCOCCOSIS.pdf

26. Öge, H., Kalinbacak, F., Gicik, Y.& Yildiz K. (1998).

The prevalence of some metacestodes (Hydatid cyst, Cysticercus tenuicollis, Cysticercus bovis) in sheep, goat and cattle slaughtered in Ankara province. Ankara Univeristesi Veteriner Fakültesi Dergisi, 45, 123-130.

27. Presnell, J. & Schreibma, M.P. (1997) Animal tissue techniques. 5th Ed, The John Hopkins University Press Ltd, London.

28. Sayır, F. & Çobanoğlu, U. (2013). Yöremizin endemik paraziter hastalığı: Kist hidatik. Van Tıp Dergisi, 20(4), 288-293.

29. Solcan, C., Solcan, G., Ionita, M., Hristescu, D.V. & Mitrea, I.L. (2010). Histological aspects of cystic echinococcosis in goats. Science Parasitology, 11,191–198.

30. Thompson, R.C.A. & Lymbery, A.J. (1995). Echinococcus and Hydatid Disease. Trans R Soc Trop Med Hyg, 89, 579.

31. Thompson, R.C.A. & McManus, D.P. (2002). Towards a taxonomic revision of the genus Echinococcus. Trends in Parasitology, 18, 452-57.

32. Tilkan, K.O., Uysal, S. & Gökçe, M. (2018). Hidatik kist hastalığı. Batı Karadeniz Tıp Dergisi, 2, 153-159.

33. Wen, H., Vuitton, L., Tuxun, T., Li, J., Vuitton, D.A., Zhang, W. & McManus, D.P. (2019). Echinococcosis: advances in the 21st century. Clinical Microbiology Reviews, 32, e00075-18.

34. Yıldız, K. & Tunçer, Ç. (2005) Kırıkkale'de sığırlarda kist hidatik'in yayılışı. Türkiye Parazitol Derg, 29, 247-250.

35. Zhang, C., Wang, L. Ali, T., Li, L., Bi, X, Wang, J., Lü, G., Wen, H & Lin, R. (2016). Hydatid cyst fluid promotes peri-cystic fibrosis in cystic echinococcosis by suppressing miR-19 expression. Parasites Vectors, 9, 278.

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Research Article

Determination of *Helicobacter heilmannii* in cats by real time polymerase chain reaction and histopathology

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ABSTRACT

In recent years, many *Helicobacter* species have been identified in domestic animals and researches on identifying these species as zoonotic agents are increasing. The aim of this study is to reveal the presence of *H.heilmannii* in stomach and liver tissues taken during necropsies of domestic, stray and shelter cats by comparing the histopathological findings and Real-time PCR results. The material of the study consisted of stomach and liver tissues taken from 41 cats who died from different causes and were necropsied. DNA of *H. heilmannii* was determined in the stomach tissues of 36 (87.8%) cats and the liver tissues of 24 (58.5%) cats in the study conducted with Real-time PCR using specific primers of *H. heilmannii*. Histopathologically, degeneration, desquamation and necrosis in the gastric epithelium, fibrosis and edema in the lamina propria, and lymphoplasmatic cell infiltration were detected in cats diagnosed with gastritis. Eight cats were positive in Hematoxylin-Eosin staining and nineteen cats were positive in the staining with Warthin Starry of the sections, in terms of Helicobacter-like microorganisms. Microscopically, dissociation in the remark cords, hydropic degeneration in hepatocytes, and focal mononuclear cell infiltrations in some sections were determined in the livers. In conclusion, the detection of *H. heilmannii* at the rate of 87.8% in cat stomachs by Real-time PCR revealed a high prevalence of *H. heilmannii* in cats in the Konya region of Turkey. In addition, it was concluded that histopathological examinations are necessary to correlate the presence of bacteria with the disease state.

INTRODUCTION

Helicobacter heilmannii is a 4 to 10 µm long spiral-shaped, three to eight helical, flagellated and motile bacterium previously known as *"Gastrospirillium hominis"* (Murray et al., 1995). According to reports, studies on cats usually identify the *H. heilmannii, H. pylori, H. felis, H. bizzozeronii,* and *H. salomonis* species (Kubota-Aizawa et al., 2017; Bruyette, 2020; Matos et al., 2020). These agents have also been identified in human gastric biopsies, and studies have been published stating that they may be zoonotic (Haesebrouck et al., 2009; Tabrizi et al., 2015; Blois, 2020). It is known that *H. heilmannii* colonizes the stomach mucosa of cats, with detection rates varying from 57% to 100% (Hong et al., 2016).

A sequence similarity of 99% was determined between *Helicobacter* species isolated from cats suffering from gastric disease and those isolated from humans (Kubota-Aizawa et al., 2017). *Helicobacter* species were positive in 40% of nodular gastritis cases in humans, 24% of MALT lymphoma cases, 17% of chronic gastritis and 33% of gastroduodenal ulcer cases (Nakamura et al., 2020). *H. heilmannii* has been detected in stomach biopsies of patients with gastric symptoms at rates ranging from 8% to 19% (Bahadori et al., 2018; Matos et al., 2020).

Although *H. heilmannii* has been associated with chronic active gastritis, its pathogenicity in cats and dogs in terms of gastritis, peptic ulceration, and chronic vomiting remains unclear (Matos et al., 2020). Researchers reported that the majority of *Helicobacter* infections in cats and dogs were asymptomatic (Otto et al., 1994; Eaton et al., 1996; Norris et al., 1999). In cases of gastritis of different severity associated with *Helicobacter* species, lymphocyte and plasma cell infiltrations with eosinophil leukocytes have been reported. It has also been described that the stomach glands were enlarged, and fibrosis was determined microscopically (Erginsoy et al., 2006). Husnik et al. (2022) stated that although *Helicobacter* species were identified during microscopic examinations of the stomachs of dogs, the presence of *Helicobacter* species was not associated with the severity of the inflammation.

Histopathology, cytology, bacterial isolation and identification, polymerase chain reaction (PCR) techniques based on the replication of the genomic DNA of the agent, and serological techniques are the main methods used in studies aiming the detection of *Helicobacter* species in cats and dogs (Happonen et al., 1996; Neiger et al., 1999). The aim of this study is to reveal by Real-time PCR and histopathological methods the presence of *H. heilmannii* in stomach and liver tissues taken during necropsy from domestic, stray and shelter cats, which died from different causes, and to compare histopathological

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findings with Real-time PCR results.

MATERIAL and METHODS

Animal materials

The stomach and liver tissues of 41 cats, who died in 2019-2022 for different reasons and were brought to the Department of Pathology, Faculty of Veterinary Medicine, Selcuk University for necropsy, constituted the material of the study. Eleven of the cats examined in the study were from the shelter, and twenty, stray cats brought in by municipal officials and animal lovers. Ten of the cats were domestic cats. Twenty-four of the studied cats were female, and seventeen were male. The related study was approved by SÜVDAMEK (Decision no: 2022/75).

Histopathological examination

Stomach and liver samples taken for histopathological examinations were fixed in a 10% buffered formalin solution, and routine tissue follow-up was performed. The obtained sections were stained with Hematoxylin-Eosin (HE) and Warthin Starry (WS) methods (Luna, 1968). The prepared sections were examined under a light microscope (Olympus BX51, Tokyo, Japan).

RESULTS

Macroscopically, bronchopneumonia was found in 9 cats, diaphragmatic hernia in 2 cats, myocarditis in 3 cats, trauma symptoms in 15 cats, and gastroenteritis in 20 cats. Microscopic examination of samples taken from the antrum region of the stomachs revealed degeneration, desquamation and necrosis of the mucosa and glandular epithelium in ten cats. In addition, lamina propria edema was observed in these cats. Mononuclear cell infiltration and fibrosis in the lamina propria were detected in four stomach samples (Figure 1.A). Eight cats were found to be positive for Helicobacter-like microorganisms in the HE staining of the sections and nineteen cats in the WS staining (Figure 1.B). Microscopic examination of liver samples were determined dissociation of remark cords and hydropic degeneration of hepatocytes in nineteen cats. Focal mononuclear cell infiltrations were observed in portal area in four of the livers examined (Figure 1.C). Helicobacter-like microorganisms could not be detected in HE and WS staining of liver sections.

DNA of *H. heilmannii* was detected in stomach by Real-time PCR in 36 cats. In the study, *H. heilmannii* rates determined by Real-time PCR in domestic, stray and shelter cats 80 % (8/10) , 90% (18/20) and 90,9% (10/11), respectively. The cats identified with *H. heilmannii* by Real-time PCR, 44.44% (16/36) were male, and 55.55% (20/36) were female. According to age, 22.22% (8/36) were younger than one year old, 44.44% (16/36) were between 1-3 years old, and 33.33% (12/36) were older than 3 years.

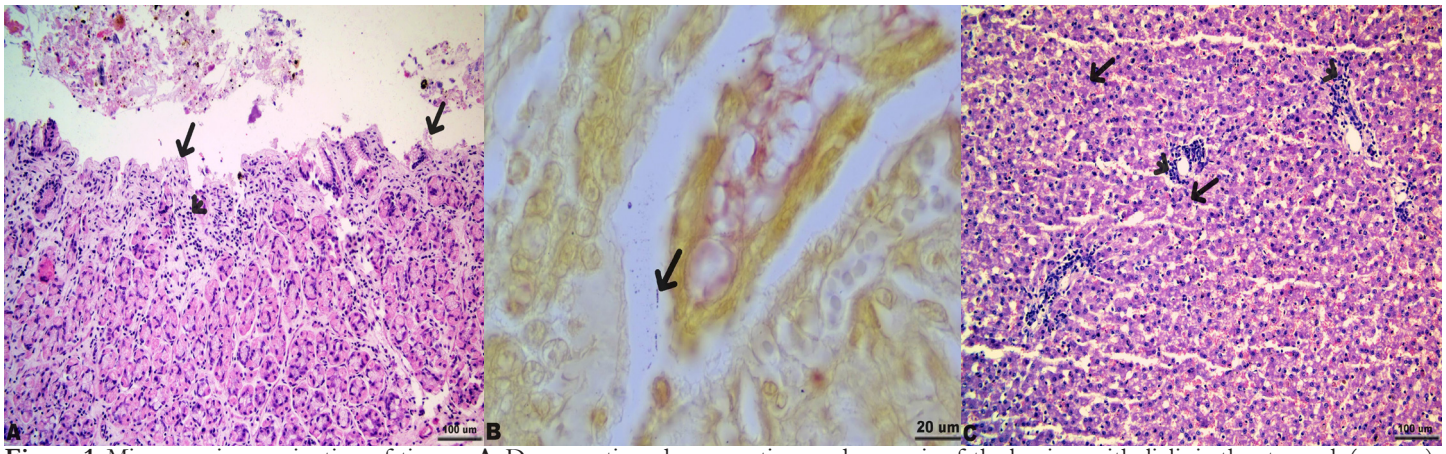

Figure 1. Microscopic examination of tissues. **A.** Degeneration, desquamation, and necrosis of the lamina epithelialis in the stomach (arrows), inflammatory cell infiltration (arrowhead), HE, x200. **B.** Helicobacter-like microorganism (arrow), WS, x1000. **C.** Hydropic degeneration (arrows) and focal inflammatory cell infiltration (arrowheads) of liver parenchyma, HE, x200.

Real-time PCR (qPCR) examination

Tissue samples taken from the antrum region of the stomach and the right lobe of the liver were stored at -20°C prior to Real-time PCR examinations. The DNA isolation from stomach and liver samples was performed using a commercial DNA isolation kit (Roche, MagNA Pure LC DNA, Cat No; 03264785001). The obtained DNAs were stored at -20°C. The DNA copies of *H. heilmannii* were detected with a Real-time PCR device (Light Cycler 2.0, Roche) using primer probes prepared by a private company. Primer sequences used in Real-time PCR analysis were F: GGG CGA TAA AGT GCG CTT G, R: CTG GTC AAT GAG AGC AGG (Neiger ve ark 1998). Deionized water was used as the negative control.

The DNA of *H. heilmannii* was detected in liver samples with Real-time PCR in 24 cats using *H. heilmannii*-specific primers (Figure 2). Real-time PCR results showed that male cats constituted 45.83% (11/24) of cats with *H. heilmannii* found in liver samples, and female cats, 54.16% (13/24). Cats with copies of *H. heilmannii* DNA in their liver 25% (6/24) were less than one year old, 41.6% (10/24) were between 1-3 years old, and 33.3% (8/24) were older than 3 years.

DNA of *H. heilmannii* was determined in the stomach of 24 cats and in the liver of four cats in the examinations per-

formed with Real-time PCR from cats whose histopathological findings could not be determined (Figure 2). DNA of *H. heilmannii* could not be detected by Real-time PCR in 4 of 12 cats with gastritis in histopathological examinations. The presence of the DNA of *H. heilmannii* was revealed by Real-time PCR in four cats with hepatitis detected in histopathological examinations.

Figure 2. Amplification curves of 24 cases found positive for H. heilmannii by real-time PCR in the liver tissues of the cats examined in the study. (PC; Positive Control, NC; Negatif Control)

DISCUSSION

There are studies conducted in both human medicine and veterinary medicine to reveal the pathology and zoonosis importance of *Helicobacter* species (Akcakavak et al., 2023; Eaton et al., 1993; Eaton et al., 1996; Happonen et al., 1998; Jalava et al., 1997; Jalava et al., 1998; Diker et al., 2002). In their study on humans, Stolte et al. (1994) determined that 111 of the 125 patients with *H. heilmannii* identified had a history of contact with one or more animals. Stolte et al. (1994), *H. heilmannii* reported that 1.6% of the patients they detected also had *H. pylori*. In studies investigating *H. heilmannii* in cats and dogs, *H. heilmannii* was determined at rates ranging from 20% to 100% (Kubota-Aizawa et al., 2017; Matos et al., 2020) and due to the fact that humans share their habitats with cats and dogs, studies have focused on cats and dogs with the suspicion that these animals may be the source of human diseases with *Helicobacter* species in the aetiology (Eaton et al., 1993; Eaton et al., 1996; Jalava et al., 1997; Jalava et al., 1998; Diker et al., 2002). The fact that DNA *H. heilmannii* was determined by Real-time PCR in 36 of 41 cats examined in this study reveals the importance of studies on the diagnosis and determination of the prevalence of *H. heilmannii* in cats.

Hermanns et al. (1995) stated that they detected Helicobacter-like organisms (HLOs) in 76% of cats in their study determining the histopathological changes in cats and dogs with HLOs. They reported that they detected degeneration in the gastric gland epithelium, neutrophil granulocyte and lymphocyte infiltration, fibrosis and edema in the lamina propria in microscopic examination. Husnik et al. (2022) reported that they detected histopathologically epithelial damage, lymphoid follicular hyperplasia, lymphocytic-plasmacytic cell infiltrations and fibrosis in *Helicobacter* positive in dogs. When the histopathological findings of our study were evaluated, they were compatible with the literature.

Erginsoy et al. (2006) determined Helicobacter-like microorganisms in the gastric mucosa of 28 of the 30 stray cats examined by the immunohistochemical method. Akhtardanesh et al. (2006) used Giemsa staining in cytology examination of stomach samples taken from stray cats in Tehran, and found the infection rate of Helicobacter-like microorganisms in the antrum and stomach body of cases as 63.2% and 77.2%, respectively. The major gastric Helicobacter strains in cats are primarily H. heilmannii and H. felis. According to reports, the prevalence of these two species in cats varies between 57% and 100% (Geyer et al., 1993; Hong et al., 2016). In this study, DNA of 87.8% (36/41) of *H. heilmannii* were detected by Real-time PCR in stomach samples of cats.

In this study, DNA of *H. heilmannii* was identified in 24 cat stomachs in Real-time PCR examinations from cats whose histopathological findings could not be determined, which supports the point of view that *H. heilmannii* may be a part of the stomach flora in cats (Gökalp & Gökalp, 2021). In the prevalence study conducted by Happonen et al (1996), they stated that they found *Helicobacter* spp at equal rates in young and old animals. In the current study, of the 36 cats whose stomach samples were identified as positive for *H. heilmannii* by Real-time PCR, 8 were younger than 1 year old, 16 were between 1-3 years old, and 12 were older than 3 years old. In this study, similar to the results determined in the stomach samples, 6 of the 24 liver samples determined positive for *H. heilmannii* belonged to cats under 1 year old, 10 of them were between 1-3 years old and 8 of them belonged to cats older than 3 years. In the light of the findings of our study, it was thought that *H.*

heilmannii could be encountered more frequently in cats aged 1-3 years and older in the Konya region.

In the study conducted by Sağnak (2007), they reported that they detected *Helicobacter* DNA in 29 (58%) of 50 male dog feces and 36 (78.3%) of 46 female dog feces and the difference between genders was statistically significant. In this study, when the distribution of *H. heilmannii* DNA copies in stomach and liver tissues is examined by gender, it is understood that it is higher in females, similar to the report of Sağnak (2007). However, since the number of samples was insufficient in this study, statistical comparisons could not be made about the relationship of *H. heilmannii* with gender in cats.

Diagnostic methods such as histopathology, cytology, culture, urea breath test and serological tests are often used in human medicine for the diagnosis of *Helicobacter* infections. However, PCR and culture tests are seen as the only methods of identifying agents at the species level (Neiger & Simpson, 2000). In this study, Helicobacter-like microorganisms were detected in 8 cats with HE staining and 19 cats with WS staining, and specific findings could not be detected in the histopathological examinations. All this has strengthened the conviction that Real-time PCR is a rapid, specific and sensitive diagnostic test that can be used to specify *H. heilmannii* in cats.

CONCLUSION

In this study, the presence of *H.heilmannii* in stomach and liver tissues taken during necropsies of domestic, stray and shelter cats that died from different causes was demonstrated by comparing the histopathological findings and Real-time PCR results. As a result, *H. heilmannii* was detected at the rate of 87.8% in cat stomachs by Real-time PCR, and it was found that *H. heilmannii* had a high prevalence in cats in the Konya region of Turkey. In addition, it was concluded that PCR analyzes are very useful in determining the agent, but histopathological examinations are necessary to associate the presence of bacteria with the disease state.

DECLARATIONS

Ethics Approval

It was approved by Selcuk University Veterinary Faculty Experimental Animal Production and Research Center Ethics Committee (02.06.2022, 2022/75).

Conflict o Interest

The authors have no conflicts of interest.

Consent for Publication

Not applicable.

Author contribution

Idea, concept and design: NT, MT, GA

Data collection and analysis: ZC, AB, RS, VK, MO

Drafting of the manuscript: NT, MT, GA

Critical review: MT, GA

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

1. Akcakavak, G., Tuzcu, M., Tuzcu, N., Celik, Z., Tural, A., & Dagar, O. (2023). Investigation with Real- Time PCR and Histopathology on the presence of H. felis, H. heilmannii and H. pylori in dogs. Revista Científica de la Facultad de Ciencias Veterinarias, 33(1), e33214, 1-7. https://doi.org/10.52973/rcfcv-e33214

2. Akhtardanesh, B., Jamshidi, S., Sasani, F., Mohammadi, M., Bokaee, S., & Zahraie Salehi, T. (2006). Helicobacter spp. infection and gastric lesions in domestic and stray cats. Veterinarski arhiv, 76(6), 479-488.

3. Bahadori, A., De Witte, C., Agin, M., De Bruyckere, S., Smet, A., Tümgör, G., Gökmen, T. G., Haesebrouck, F., & Köksal, F. (2018). Presence of gastric Helicobacter species in children suffering from gastric disorders in Southern Turkey. Helicobacter, 23(5), e12511. https://doi.org/ 10.1111/hel.12511

4. Blois, S. (2020). Helicobacter infection in small animals. MSD Manual Veterinary Manual.

5. Bruyette, D. (2020). Clinical Small Animal Internal Medicine. John Wiley & Sons.

6. Diker, K. S., Haziroğlu, R., Akan, M., Çelik, S., & Kabakçı, N. (2002). The prevalence, colonization sites and pathological effects of gastric helicobacters in dogs. Turkish journal of veterinary & animal sciences, 26(2), 345-351.

7. Eaton, K., Dewhirst, F., Radin, M. J., Fox, J., Paster, B., Krakowka, S., & Morgan, D. (1993). Helicobacter acinonyx sp. nov., isolated from cheetahs with gastritis. International journal of systematic and evolutionary microbiology, 43(1), 99-106. https://doi.org/10.1099/00207713-43-1-99.

8. Eaton, K., Dewhirst, F., Paster, B., Tzellas, N., Coleman, B., Paola, J., & Sherding, R. (1996). Prevalence and varieties of Helicobacter species in dogs from random sources and pet dogs: animal and public health implications. Journal of Clinical Microbiology, 34(12), 3165-3170. https://doi.org/10.1128/jcm.34.12.3165-3170.1996

9. Erginsoy, S., Sözmen, M., & Özcan, K. (2006). Gastric Helicobacter-like organisms in stray cats. Acta Veterinaria Brno, 75(1), 91-98. https://doi.org/10.2754/avb200675010091

10. Geyer, C., Colbatzky, F., Lechner, J., & Hermanns W (1993). Occurrence of spiral-shaped bacteria in gastric biopsies of dogs and cats. Veterinary Record, 133(1), 18-19

11. Gökalp, G., & Gökalp, A. B. (2021). Kedilerin Önemli Bakteriyel Zoonozu; Helicobacter Heilmannii. Journal of Anatolian Environmental and Animal Sciences, 6(3), 333-340. https://doi.org/10.35229/jaes.891405

12. Haesebrouck, F., Pasmans, F., Flahou, B., Chiers, K.,

Baele, M., Meyns, T., Decostere, A., & Ducatelle, R. (2009). Gastric helicobacters in domestic animals and nonhuman primates and their significance for human health. Clinical microbiology reviews, 22(2), 202-223. https://doi.org/10.1128/CMR.00041-08.

13. Happonen, I., Saari, S., Castren, L., Tyni, O., Hänninen, M. L., & Westermarck, E. (1996). Occurrence and topographical mapping of gastric Helicobacter□ like organisms and their association with histological changes in apparently healthy dogs and cats. Journal of Veterinary Medicine Series A, 43(1□ 10), 305-315. https://doi.org/10.1111/j.1439-0442.1996.tb00457.x.

14. Happonen, I., Linden, J., Saari, S., Karjalainen, M., Hänninen, M.-L., Jalava, K., & Westermarck, E. (1998). Detection and effects of helicobacters in healthy dogs and dogs with signs of gastritis. Journal of the American Veterinary Medical Association, 213(12), 1767-1774.

15. Hermanns, W., Kregel, K., Breuer, W., & Lechner, J. (1995). Helicobacter-like organisms: histopathological examination of gastric biopsies from dogs and cats. Journal of comparative pathology, 112(3), 307-318. https://doi.org/10.1016/s0021-9975(05)80083-0.

16. Hong, S., Chung, Y., Kang, W.-G., & Kim, O. (2016). Detection of Helicobacter felis in a cat with gastric disease in laboratory animal facility. Laboratory Animal Research, 32(2), 122-127. https://doi.org/10.5625/lar.2016.32.2.122

17. Husnik, R., Klimes, J., Kovarikova, S., & Kolorz, M. (2022). Helicobacter Species and Their Association with Gastric Pathology in a Cohort of Dogs with Chronic Gastrointestinal Signs. Animals, 12(10), 1254. https://doi.org/10.3390/ani12101254

18. Jalava, K., Kaartinen, M., Utriainen, M., Happonen, I., & Hänninen, M.-L. (1997). Helicobacter salomonis sp. nov., a canine gastric Helicobacter sp. related to Helicobacter felis and Helicobacter bizzozeronii. International journal of systematic and evolutionary microbiology, 47(4), 975-982. https://doi.org/10.1099/00207713-47-4-975

19. Jalava, K., On, S. L., Vandamme, P. A., Happonen, I., Sukura, A., & Ha□ nninen, M.-L. (1998). Isolation and identification of Helicobacter spp. from canine and feline gastric mucosa. Applied and Environmental Microbiology, 64(10), 3998-4006.

20. Kubota-Aizawa, S., Ohno, K., Kanemoto, H., Nakashima, K., Fukushima, K., Uchida, K., Chambers, J. K., Goto-Koshino, Y., Mimuro, H., & Watanabe, T. (2017). Epidemiological study on feline gastric Helicobacter spp. in Japan. Journal of veterinary medical science, 16-0567. https://doi.org/10.1292/jvms.16-0567.

21. Luna, L. G. (1968). Manual of histologic staining methods of the Armed Forces Institute of Pathology, McGraw-Hill.

22. Matos, R., De Witte, C., Smet, A., Berlamont, H., De Bruyckere, S., Amorim, I., Gärtner, F., & Haesebrouck, F. (2020). Antimicrobial susceptibility pattern of Helicobacter heilmannii and Helicobacter ailurogastricus isolates. Microorganisms, 8(6), 957.

23. Murray, P. R., Baron, E. J., Pfaller, M. A., Tenover, F. C., & Olken, R. H. (1995). Manual of Clinical Microbiology (6th Ed.). ASM Press, Washington, USA, 1247-1256.

24. Nakamura, M., Øverby, A., Michimae, H., Matsui, H., Takahashi, S., Mabe, K., Shimoyama, T., Sasaki, M., Terao, S., & Kamada, T. (2020). PCR analysis and specific immunohistochemistry revealing a high prevalence of non□ Helicobacter pylori Helicobacters in Helicobacter pylori□ negative gastric disease patients in Japan: High susceptibility to an Hp eradication regimen. Helicobacter, 25(5), e12700. https://doi.org/10.1111/hel.12700

25. Neiger, R., Dieterich, C., Burnens, A., Waldvogel, A., Corthésy-Theulaz, I., Halter, F., Lauterburg, B., & Schmassmann, A. (1998). Detection and prevalence of Helicobacter infection in pet cats. Journal of Clinical Microbiology, 36(3), 634-637.

26. Neiger, R., Tschudi, M. E., Burnens, A., Goke, B., & Schmassmann, A. (1999). Diagnosis and identification of gastric Helicobacter species by polymerase chain reaction in dogs. Microbial Ecology in Health and Disease, 11(4), 234-240. https://doi.org/10.1080/089106099435682

27. Neiger, R., & Simpson, K. W. (2000). Helicobacter infection in dogs and cats: facts and fiction. Journal of Veterinary Internal Medicine, 14(2), 125-133. https://doi.org/10.1892/0891-6640(2000)014<0125:iidacf>2.3.co;2.

28. Norris, C., Marks, S. L., Eaton, K., Torabian, S., Munn, R., & Solnick, J. V. (1999). Healthy cats are commonly colonized with "Helicobacter heilmannii" that is associated with minimal gastritis. Journal of Clinical Microbiology, 37(1), 189-194.

29. Otto, G., Hazell, S., Fox, J., Howlett, C., Murphy, J., O'Rourke, J., & Lee, A. (1994). Animal and public health implications of gastric colonization of cats by Helicobacter-like organisms. Journal of Clinical Microbiology, 32(4), 1043-1049. https://doi.org/10.1128/jcm.32.4.1043-1049.1994

30. Sağnak, N. S. (2007). İstanbul Yöresinde Sağlıklı ve Klinik Belirti Gösteren Köpeklerde Helicobacter pylori Varlığının Kültür, Polimerase Chain Reaction (PCR) ve Restriction Fragment Length Polymorphism (RFLP) ile Saptanması, Doktora Tezi, İ.Ü., Sağlık Bilimleri Enstitüsü.

31. Stolte, M., Wellens, E., Bethke, B., Ritter, M., & Eidt, H. (1994). Helicobacter heilmannii (formerly Gastrospirillum hominis) gastritis: an infection transmitted by animals? Scandinavian Journal of Gastroenterology, 29(12), 1061-1064. https://doi.org/10.3109/00365529409094888.

32. Tabrizi, A. S., Derakhshandeh, A., Esfandiari, A., & Atashi, Z. A. (2015). Identification of Helicobacter spp. in gastrointestinal tract, pancreas and hepatobiliary system of stray cats. Iranian Journal of Veterinary Research, 16(4), 374.

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Research Article

Total antioxidant, total oxidant and oxidative stress levels in free-living birds

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ABSTRACT

Antioxidants provide protection against free radicals formed as a result of increased metabolism in living organisms and the damage these radicals cause to the cell. Determining the antioxidant levels can help eco-physiologists in the field in understanding the physiological state of the animal at that moment and in conservation biology. In this study, TOC (Total Oxidant Capacity), TAC (Total Antioxidant Capacity) and OSI (Oxidative Stress Index) values of 12 Long-legged Buzzards (Buteo rufinus), 7 Common Buzzards (Buteo buteo) and 6 Golden Eagles (Aquila chrysaetos), 15 Grey Herons (Ardea cinerea), 7 Eurasian Eagle Owls (Bubo bubo) brought to rehabilitation centre with injuries due to various reasons were examined. The birds brought to the center were first examined physically. Species-specific rooms were kept until each bird had recovered. After being rehabilitated, blood was taken from the birds 1-2 days before being released into the wild. As a result of blood analysis, TOC and OSI values were found to be high in Eurasian Eagle Owl, Golden Eagle, Long-legged Buzzard and Common Buzzards which were brought with a diagnosis of gunshot wounds, soft tissue trauma, femur or wing fractures. Although these birds had been rehabilitated, the reason for the high TOC and OSI values in individuals with these diagnoses may be the trauma experienced by these free-living birds in nature and then being held in captivity. After the birds were treated and rehabilitated, they were released back to nature in habitats specific to each species.

INTRODUCTION

The habitats of wild birds living freely in nature are open areas that can be a few kilometers or thousands of kilometers. Therefore, keeping these animals under captivity for various reasons may cause an increase in their metabolism and stress as a result of this (Coles, 2007; Cohen et al., 2008; Fischer & Romero, 2018). In addition, the sources of nutrient they feed in nature or the contamination of these sources for various reasons may change the levels of antioxidants and oxidants in their bodies. This can also be a source of extra stress to the animal (Costantini & Verhulst, 2009; Espín et al., 2014; Abbasi et al., 2017). In nature, wild birds, especially raptors are more sensitive to environmental influences than many birds. Human interventions to the habitats of wild free-living birds in nature make it difficult for these animals to survive and even put many species of birds to the category of endangered species (Coles, 2007). According to the data we have, it is stated that 65% of the birds are brought to rehabilitation centres due to traumatic injuries caused by human impact, while 85% are brought due to human-related deaths (Fix & Barrows, 1990; Desmarchelier et al., 2010). A significant number of wild birds are brought to rehabilitation centres due to reasons such as traffic accidents, pesticide intoxication, gunshot wounds, hitting electric wires or high voltage lines (Desmarchelier et al., 2010; Malik & Valentine, 2018). While these free-living animals already have stress caused by their natural enemies, human pressure also causes this stress to increase. As a result of this, the metabolism of these birds may increase and their oxidant and antioxidant states may change. The balance between oxidant production and antioxidant defense is considered an important indicator of an individual's health, and the individual oxidative stress level is the best indicator of this (Costantini et al., 2006). Antioxidant capacity of all living organisms is among the factors that affect their longevity and plays a very important role in their physiology. Agents that prevent the oxidation caused by free radicals and that have the ability to capture and stabilize free radicals are called 'antioxidant' (Şahin et al., 2015). Any negative effect that occurs in the organism can be prevented by antioxidants. The main task of antioxidants is to keep the oxygen in the existing environment and to prevent the initiation or further progress of oxidation reactions. When free radicals exceed the physiological levels in the cell, they cannot be destroyed by antioxidants. In this case, oxidative stress occurs. Disruption of the balance between oxidants and antioxidants at cellular level is defined as oxidative stress. In this process, as a result of lipid peroxidation caused by free radicals, phospholipids in the membrane are oxidized and as a result, membrane permeability increases. As a result, the ion balance in the cell is disrupted, functions of membrane-bound surface receptors are disrupted and many biochemical functions are not fulfilled (Mis et al., 2018).

Wild birds are very sensitive to environmental changes and

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any changes that may occur in their environment cause significant physiological responses due to the stress they experience. As a result of these reactions, the animal transfers its existing resources predominantly to survival, which puts an extra physiological burden on the animal (Costantini & Verhulst, 2009; Cram et al., 2015; Fischer & Romero, 2018). Reasons such as smell, sound, light conditions and daily diet in rehabilitation centres may cause wild birds to get extra stressed (Morgan & Tromborg, 2007; Fischer & Romero, 2018).

Therefore, understanding the responses of wild birds to these stressors, their adaptation mechanisms, which strategies are used and the differences between species is very important in terms of conservation biology (Fischer & Romero, 2018). Not only clinical examination, but also determination and evaluation of hematological, biochemical and physiological anomalies are very important in the success of such survival and conservation strategies (Black et al., 2011). In the rehabilitation of wild raptors, it is also very important to determine when the bird will be considered suitable and healthy to be released, that is, the release criteria.

There are a large number of studies in literature on TOC (Total Oxidant Capacity), TAC (Total Antioxidant Capacity) and OSI (Oxidative Stress Index) values showing the stress states of broilers and wild birds kept in captivity in cages for a long time (Mis et al., 2018; Wang et al., 2021). In recent years, studies on oxidative stress index, antioxidant levels, DNA damage of wild birds living free in nature have gained momentum. Based on previously conducted studies, the expected hypothesis of the present study is that oxidative stress index will increase especially in birds which are injured by gunshot fire, which have fractures and bone tissue trauma (Yaprakci et al., 2016). However, considering that the birds are rehabilitated, it is a question mark whether these values as a result of rehabilitation will reflect the results of normal and healthy individuals or whether they will be high due to the stress they increase, the presence of trauma and humans. For this reason, blood samples were taken from free-living wild birds brought to the centre due to various reasons and their TOC, TAC and OSI data were evaluated.

MATERIAL and METHODS

Kafkas University/Kafkas Wild Animal Protection, Rescue, Rehabilitation Application and Research Centre

Kafkas University/Kafkas Wild Animal Protection, Rescue, Rehabilitation Application and Research Centre located in Kars in Türkiye is a center where exhausted or injured wild animals animals in the Eastern Anatolia and Black Sea regions are brought to be rehabilitated. There are administrative, clinical and rehabilitation departments in the center in the Kafkas University campus, which serves under the 13 Regional Directorates of Nature Conservation and National Parks of the Ministry of Agriculture and Forestry. Many wild animals such as brown bear, lynx, fox, wolf, roe deer, mountain goat, golden eagle, red hawk and eagle are brought to the center, which are injured for various reasons. After these animals are treated, they are released back into nature. The center serves provinces of Erzurum, Gümüşhane, Erzincan, Bingöl, Bayburt, Artvin, Ardahan, Ağrı and Iğdır. Since 2020, 380 wild animals have been brought to the center, nearly 200 of them were cured and released into their natural environment.

Animal material

In this study, TOC, TAC and OSI values of 12 Long-legged buzzard (*Buteo rufinu*), 7 Common buzzard (*Buteo buteo*), 6 Golden eagle (*Aquila chrysaetos*), 15 Grey heron (*Ardea cinerea*) and 7 Eurasian eagle-owl (*Bubo bubo*) brought to Kafkas University/Kafkas Wild Animal Protection, Rescue, Rehabilitation Application and Research Centre were examined. All Grey herons were brought to the rehabilitation centre because the trees they nested in fell down during storm and they could not leave the nest. No problems were found in their examination and the diagnosis was fatigue due to dehydration. Other birds were injured in nature due to various reasons and they were randomly brought to rehabilitation centre both by citizens and by wild life protection teams. All birds brought to the rehabilitation center were adults.

Table 1 and 2 show the reasons why all of the birds were brought to the centre. All of the birds in the centre were kept for 1-3 months in specially arranged, isolated bird care rooms with an international size and standards and then in flight tunnels suitable for the species (5m/6m/30m). To avoid stress, they were not disturbed for any reason other than the routine visits of researchers. They were fed with red or white meat specific to the species for 6 days a week. Food and water were given *ad libitum*. Since the important thing in rehabilitation and conservation biology is to treat the animal and return it to wild life in a healthy way, no unnecessary medical intervention was performed during treatment process on birds which were brought injured, sick or destitute to the centre. In their first admission to the centre, no blood sample was taken from these birds for examination. Due to these medical reasons, we do not have any control group data that show the initial disease status of the birds in our study. 1-2 days before the birds to be released into the wild, blood samples were taken and all individuals were released back to wild life in suitable sites.

Obtaining blood samples and analyses

Blood was taken from the wing ulnar/basilic vena of the birds brought to the rehabilitation centre and transferred to EDTA (BD vacutainer, K_2 EDTA) tubes; after they were centrifuged for 5 minutes at 3000 r.p.m. (Electro-mag M815 M), the plasma samples were transferred to clean tubes. The plasma samples were kept in a deep freezer (Profilo 6600) at - 20 °C until they were analyzed.

TOC and TAC Analysis of blood samples

TOC and TAC measurements (Rel Assay Diagnostics) were carried out with colorimetric test according to the recommended procedure.

TOC measurement

Plasma total oxidant capacity is measured with the ferric ion forming a color intensity based on the ration of oxidants in the environment as a result of creating a colorful complex

with acidic chromogenic and spectrophotometric determination of this. Its principle is based on the oxidation of oxidants present in the plasma to iron ion with iron on-chelate complex. Based on this principle, the measurement was made in accordance with the procedure in commercial test kits. A commercial kit was used for the determination of TOC (Rel Assay Diagnostics®) (Acke et. al., 2015). The initial solution containing reagent 1 (assay buffer) and the sample or standard was read at 530 nm for the first absorbance value. After that, prochromogen solution was added and incubated for 10 min at room temperature or 5 min at 37° C to produce colour complexing between the ferric ions and chromogen which can be measured spectrophotometrically and related to the total oxidant concentration. After incubation, the solution was read again at 530 nm (DAS Plate Reader) (Allam & Lemcke, 1975).

TAC measurement

Measurement of plasma total antioxidant capacity is determined on the basis that antioxidants in the sample reduce the dark blue-green procromogen 2,2'-azinobis (3-etilbenzotiyazolin-6-sülfonik acid) (ABTS) radical to colorless ABTS form. Based on this principle, the measurement was made in accordance with the procedure in commercial test kits. For the measurement of TAC (Rel Assay Diagnostics®) (Acke et. al., 2015) the initial solution contained reagent 1 (assay buffer) and the sample or standard absorbance was read at 660 nm for the first value. After that, ABTS radical solution was added and the mixture was incubated for 10 min at room temperature or 5 min at 37° C to allow the antioxidants to reduce the dark blue-green ABTS to form colourless, reduced ABTS before the absorbance was read again at 660 nm (DAS Plate Reader) (Allam & Lemcke, 1975).

The calculation of OSI

In order to calculate OSI, which is an indicator of oxidative stress degree, TOC value was divided by TAC value and multiplied with 100.

$$OSI = TOC / TAC \times 100$$

Analyses of data

The data were analysed with SPSS 22.0 (Statistical Package for Social Sciences). The normality test of the data was performed using the Shapiro-Wilk test, and it was found that the the all bird groups were not normally distributed separately ($p<0.05$). Statistical analysis of blood samples taken from rehabilitated birds was performed by Chi-Square test and $p \leq 0.05$ was considered as significant. Chi-Square tests of goodness of fit was used in single group comparisons. The purpose of this test; is to investigate whether a sample of *n* volumes drawn from the population is representative of the population. In this study, we tested the suitability of TOC, TAC and OSI values obtained from each bird species to the population (Table 1-5). For example, a comparison was made in the TAC values for the Eurasian eagle owls group of 7 individuals. Likewise, TOC and OSI values were also compared separately.

We used the Kruskal-Wallis and Mann-Whitney test to separately compare the TOC, TAC and OSI values in birds. For example, we compared the TAC of Eurasian eagle owls, Golden eagles and Grey herons (only 3 birds). Then we compared the TOC and OSI values for the same birds separately. We used Kruskal-Wallis test (non-parametric equivalent of One-way ANOVA) to make these comparisons. For the Long legged buzzard and Common buzzard (only 2 birds), we used the Mann-Whitney U test, which is a pairwise comparative test (Figures 1, 2 and 3).

Table 1. Plasma TAC, TOC and OSI values of rehabilitated Eurasion eagle-owl and the reasons why they were brougth to the clinic

	TAC (mmol Trolox Equv./L)	**TOC (μmol H_2O_2 Equv./L)**	**OSI (Arbitrary Unit)**	**Reason**
1	1.77	46.78	2.64	Weakness
2	1.55	262.00	16.90	Weakness
3	0.92	108.15	11.75	Left leg atrophy, neural
4	1.74	440.09	25.29	Weakness
5	0.17	44.75	26.32	Weakness
6	1.65	924.00	56.00	Shotgun Fire
7	0.46	836.36	18.18	Weakness
Mean±S.E.	**1.18±0.25**	**380.30±139.64**	**22.44±6.37**	

There is no statistical difference in TAC values ($p>0.05$). There are statistical difference in TOC and OSI values ($p<0.05$) (n=7, TAC: Chi Square= 0.444, df= 4, p= 0.979; TOC: Chi Square= 2152.312, df= 6, p= 0.000; OSI: Chi Square= 74.981, df= 6, p= 0.000). S.E.= Standart Error.

RESULTS

Table 1-5 show TOC, TAC and OSI values of all wild birds which were injured or tired for various reasons and the reasons why they were brought to rehabilitation centre, while Figure 1, 2 and 3 show the mean TOC, TAC and OSI values of birds.

As seen in Table 1, there was no statistical difference in plas-

Table 2. Plasma TAC, TOC and OSI values of rehabilitated Golden eagle and the reasons why they were brougth to the clinic

	TAC (mmol Trolox Equv./L)	TOC (μmol H_2O_2 Equv./L)	OSI (Arbitrary Unit)	Reason
1	0.49	375.75	76.68	Weakness
2	0.57	281.58	49.40	Weakness, cachexia
3	0.89	502.56	56.46	Weakness
4	1.96	46.52	2.37	Weakness
5	0.31	47.55	15.33	Shotgun fire, wing fracture
6	1.76	507.22	28.81	Shotgun fire, wing fracture
Mean±S.E.	**0.99±0.28**	**293.53±85.20**	**38.17±11.30**	

There is no statistical difference in TAC values ($p>0.05$). There are statistical difference in TOC and OSI values ($p<0.05$) (n= 6, TAC: Chi Square= 0.667, df= 3, p= 0.881; TOC: Chi Square= 737.968, df= 5, p= 0.000; OSI: Chi Square= 101.895, df= 5, p= 0.000). S.E.= Standart Error.

Table 3. Plasma TAC, TOC and OSI values of rehabilitated Grey heron and the reasons why they were brougth to the clinic

	TAC (mmol Trolox Equv./L)	TOC (μmol H_2O_2 Equv./L)	OSI (Arbitrary Unit)	Reason
1	0.69	105.36	15.26	Weakness
2	1.41	68.06	4.82	Weakness
3	1.41	18.39	1.30	Weakness
4	0.62	90.44	14.58	Weakness
5	0.48	151.98	31.66	Weakness
6	0.53	63.40	11.96	Weakness
7	0.70	32.63	4.66	Weakness
8	0.45	42.89	9.53	Weakness
9	0.23	54.07	1.24	Weakness
10	1.43	96.03	6.71	Weakness
11	1.47	15.85	1.07	Weakness
12	0.70	14.91	2.13	Weakness
13	1.58	55.94	3.54	Weakness
14	0.14	13.72	9.80	Weakness
15	0.59	41.02	6.95	Weakness
Mean±S.E.	**0.82±0.12**	**57.64±10.24**	**8.34±2.06**	

There is no statistical difference in TAC values ($p>0.05$). There are statistical difference in TOC and OSI values ($p<0.05$) (n= 15, TAC: Chi Square= 1.500, df= 8, p= 0.993; TOC: Chi Square= 380.965, df=14, p= 0.000; OSI= Chi Square: 107.921, df=14, p= 0.000). S.E.= Standart Error.

ma TAC values in Eurasian eagle-owls. On the other hand, there was a statistical difference in TOC and OSI values, and the highest TOC and OSI values were seen in Eurasian eagle-owls (No. 6) injured with gunshot fire. Similarly, there was no difference in the TAC values in the Table 2, while there are differences in the TOC and OSI values. In Golden eagles, the highest plasma TOC and OSI values (No: 1, 3 and 6) were seen in the birds that was brought with weakness, shotgun fire and wing fracture. Although OSI value was high in individual injured with gunshot fire (No. 6), they was not as high as those in the Golden eagles brought with weakness (No. 1 and 3). As a result of the statistical analysis, no differences were found in the plasma TAC values of Grey herons, but there were statistical differences in TOC and OSI Table 3.

While there was no statistical difference in TAC values in

Table 4. Plasma TAC, TOC and OSI values of rehabilitated Long-legged buzzard and the reasons why they were brougth to the clinic

	TAC (mmol Trolox Equv./L)	TOC (µmol H_2O_2 Equv./L)	OSI (Arbitrary Unit)	Reason
1	0.82	526.80	64.24	Bone tissue trauma without fractures
2	1.89	176.22	9.32	Soft tissue trauma
3	0.93	589.27	63.36	Soft tissue trauma
4	0.42	71.79	17.09	Soft tissue trauma
5	1.68	147.31	8.76	Soft tissue trauma
6	1.73	77.38	4.47	Soft tissue trauma
7	1.33	408.39	30.70	Femur fracture
8	1.02	151.04	14.80	Left claw paralysis
9	1.73	715.15	41.33	Closed wing fracture
10	1.08	841.02	77.87	Closed wing fracture
11	1.37	367.36	26.81	Closed wing fracture
12	1.19	680.65	57.19	Closed wing fracture

There is no statistical difference in TAC values ($p>0.05$). There are statistical difference in TOC and OSI values ($p<0.05$) (n= 12, TAC: Chi Square= 5.667, df= 9, p= 0.773; TOC: Chi Square= 2054.899, df= 11, p= 0.000; OSI: Chi Square= 205.559, df= 11, p= 0.000). S.E.= Standart Error.

Table 5. Plasma TAC, TOC and OSI values of rehabilitated Common buzzard and the reasons why they were brougth to the clinic

	TAC (mmol Trolox Equv./L)	TOC (µmol H_2O_2 Equv./L)	OSI (Arbitrary Unit)	Reason
1	0.88	11.18	1.27	Bone tissue trauma without fractures
2	0.70	139.86	19.98	Soft tissue trauma
3	0.82	470.86	57.42	Compound fracture
4	0.66	453.14	68.67	Femur fracture
5	1.79	390.67	21.82	Weakness
6	1.67	97.90	5.86	Weakness
7	1.75	411.18	23.49	Weakness

There is no statistical difference in TAC values ($p>0.05$). There are statistical difference in TOC and OSI values ($p<0.05$) (n= 7, TAC: Chi Square= 1.200, df= 6, p= 0.997; TOC: Chi Square= 783.096, df= 6, p= 0.000; OSI: Chi Square= 136.444, df= 6, p= 0.000). S.E.= Standart Error.

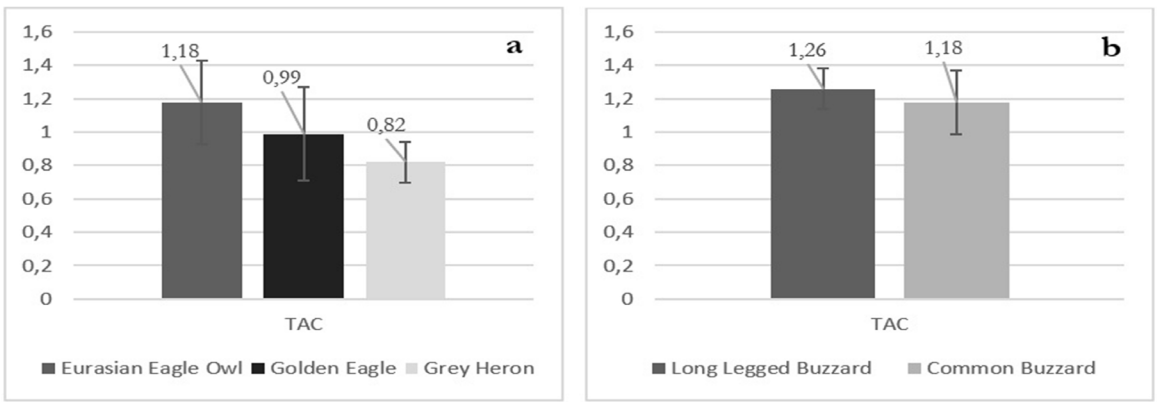

Figure 1. Figure 1.a. Mean plasma TAC (mmol Trolox Equv./L) data of Eurasion Eagle Owl, Golden Eagle and Grey Heron (Kruskal-Wallis H= 1.730, df= 2, p= 0.421). Figure 1.b. Mean plasma TAC (mmol Trolox Equv./L) data of Long Legged Buzzard, and Common Buzzard (Mann-Whitney U= 35.500, p= 0.582), $p>0.05$

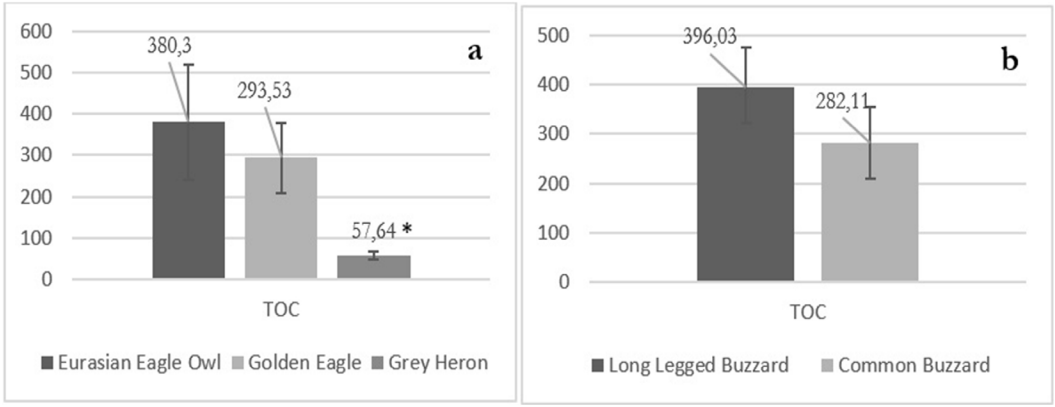

Figure 2. Mean plasma TOC (µmol H2O2 Equv./L) data of Eurasion Eagle Owl, Golden Eagle and Grey Heron (Kruskal-Wallis H= 8.831, df= 2, p= 0.012), * means statistically different, p<0.05. Figure 2.b. Mean plasma TOC (µmol H2O2 Equv./L) data of Long Legged Buzzard, and Common Buzzard (Mann-Whitney U= 31.000, p= 0.353)

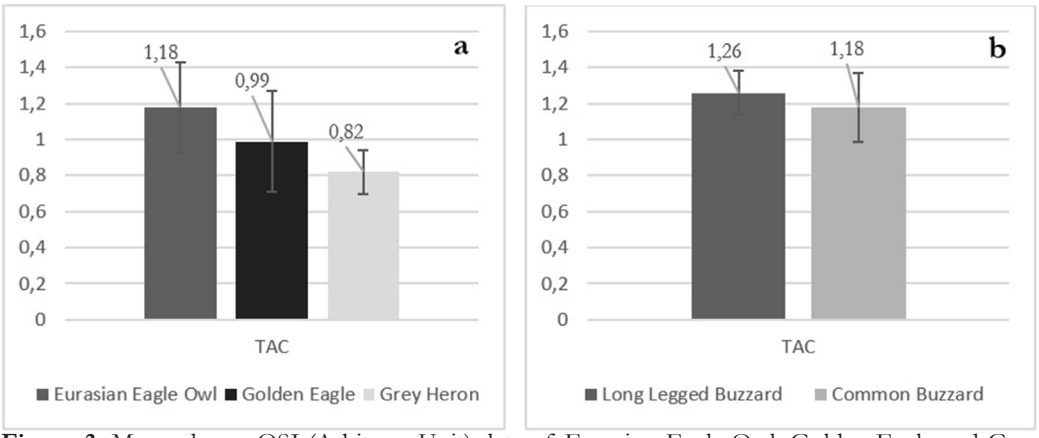

Figure 3. Mean plasma OSI (Arbitrary Unit) data of Eurasion Eagle Owl, Golden Eagle and Grey Heron (Kruskal-Wallis H= 9.45, df= 2, p= 0.09), * means statistically different, p<0.05. Figure 3.b. Mean plasma OSI (Arbitrary Unit) data of Long Legged Buzzard and Common Buzzard (Mann-Whitney U= 36.000, p= 0.612)

Long-legged buzzards and Common buzzards, there were differences in TOC and OSI Table 4 and 5. In terms of rehabilitated Long-legged buzzards, the highest plasma TOC and OSI values were found in the individual numbered 10 which was brought with closed fracture. In individuals which presented with closed fractures, OSI values are quite high. These values were found to be high in individual with the number 1 which was brought with unfractured bone trauma. OSI values were found to be high in all Long-legged buzzards except for those which were brought for soft tissue trauma (No. 2, 4, 5, 6). TOC and OSI values in all Common buzzards were high except for individuals numbered 1 and 6. Plasma TOC and OSI values were very high especially in individuals that were brought with compound fracture (No. 3) and femur fracture (No. 4).

DISCUSSION

Considering the plasma TOC, TAC and OSI values of all birds were examined, while TOC and OSI values were significantly high in all bird species except Grey heron, we believe it is necessary to draw attention to the fact that all of the Grey herons were brought with a diagnosis of weakness. Other birds were brought to the centre due to various and important traumatic reasons except for weakness.

The purpose of wild animal rehabilitation is treating the animals and releasing them to nature again. For this reason, care was taken to ensure that there is no different cause that may stress animals during the time they stayed at the centre, based on international practice standards (Redig, 1978; Chaplin et al., 1993). For example, according to international standards, birds in captivity should be kept in dark and quiet rooms so that they do not experience extra stress, they should not be disturbed, and the number of visitors should be kept to a minimum (Mander et al., 2003). The aim of rehabilitation of wild animals is to treat animals and release them back to natural life. Considering that all the birds evaluated in the study lived under relative captivity and the fact that the procedure of blood measurement may create stress, the birds were contacted each time by the same physicians and staff only when necessary for minimum stress conditions, no physical contact was experienced during the process and the same medical staff collected blood (Cooper, 1972; Campbell, 2012). In a study they conducted

with *Falco tinnuculus,* Costantini and Dell'Omo (2006) suggested that especially OXY (total serum antioxidant level) was more affected by environmental components and the changes in OXY were due to antioxidants they took with nutrients. In a study they conducted with free-living *F. tinnunculus* during the breeding time, Costantini et al. (2006) found that stress levels increased due to decreased access to nutrient in larger hatch with more sibling rivalry. In the light of this information, starting from the moment they were accepted in the centre, the animals were fed sufficiently with natural food that is completely species-specific, similar to their hunt in natural life.

It is known that free radicals and oxidative stress in cells cause many disorders such as premature aging, reproductive capacity, arthritis, cardiovascular diseases and allergic reactions. Many things can also cause increased oxidative stress in wild life. Environmental variables (Costantini & Dell'Omo, 2006; Wang et al., 2021), predator and human pressure, poachers (Yaprakci et al., 2016), nutrition (Costantini et al., 2007; Mis et al., 2018), contamination of nutrients and water sources with chemicals, radiation (Fernie & Bird 2001; Fernie & Reynolds, 2005; Abbasi et al., 2017), whether the animals is in migration period (Arnold et al., 2010), size of hatch and nest (number of young birds) (Costantini et al., 2007) are among reasons that cause free radicals and oxidative stress index in animals to increase. Studies on oxidative stress, which has a direct effect on health and life capacity by being affected by many factors, have been increasing and becoming more important in recent years (Casagrande et al., 2011; Bize et al., 2014). Metabolic rate, diseases or injuries also cause the production of abundant reactive oxygen species. These reactive oxygen species may cause oxidative damage of biomolecules and accumulation of damage, resulting in disruption of homeostatic regulation mechanism and decrease in the longevity of the animal or death (Costantini & Verhulst, 2009; Yaprakci et al., 2016). At the same time, the assessment of oxidative stress levels may be a scale in evaluating immunity status (Cram et al., 2015). In this study, TOC and OSI values in Eurasian eagle-owls, Golden eagles, Long-legged buzzards and Common buzzards brought to the rehabilitation centre with diagnoses of gunshot wound, soft tissue trauma, femur and wing fractures were relatively higher than those of the other individuals. Although these raptors were rehabilitated, the reason why TOC and OSI values were high in individuals that were brought with trauma may probably be the fact that these birds were kept in captivity after the trauma/injury these individuals experienced. Similar to the results of this study, in a study conducted on common buzzards (*Buteo* species) with gunshot wound, TOC levels were found to be much higher than the control group (fatigue, thirst, weakness, simple bruise) (Yaprakci et al., 2016). An insignificant difference was found in TAC levels when compared with the control group. In this study, high OSI levels especially in individuals that were brought with gunshot wounds, tissue trauma and fractures shows the oxidative stress due to damage in all birds. Raptors may also be stressed due to poachers or human pressure in the environment (Finkel & Holbrook, 2000; Vágási et al., 2019). Birds in nests may also be exposed to an extra fracture or trauma when they are shot by hunters from a distance or due to falling from high. All these events may increase the metabolic rate and oxidative stress of animals.

CONCLUSION

In this study, the TOC, TAC and OSI levels of Eurasian eagle-owl, Golden eagle, Long-legged buzzard, Common buzzard and Grey herons that were brought to rehabilitation centre due to various reasons were evaluated. It was thought that the high oxidative stress index of especially birds that were brought to centre due to gunshot wounds, soft tissue trauma or fractures may be due to traumas experienced by these birds. Although blood collection and other procedures were performed by the same stuff in the rehabilitation centre so that the birds would not be stressed, the fact that they could be stressed and thus their metabolism increased due to presence of humans and the fact that they were kept under captivity was presented for the attention of readers by the authors. In addition, the data we reported may not completely represent the values expected for wild free-living birds due to individual effects of trauma, nutrition and captivity stress. Due to the small number of subjects obtained through completely natural means and those brought to a specific rehabilitation centre, it was thought that the data presented here may be evaluated as pre-data for studies to be conducted with higher number of subjects. It was concluded that conducting long-term studies with a higher number of subjects in the future will contribute to a stronger demonstration of these values.

DECLARATIONS

Ethics Approval

All procedures were approved by the Kafkas University Local Ethics Committee for Animal Experiments (KAÜ-HADYEK/2019-134) and the Republic of Turkey Ministry of Agriculture and Forestry, General Directorate of Nature Conservation and National Parks (14.11.2019/21264211-288.04-E.3469713).

Conflict o Interest

The authors declare no conflict of interest.

Consent for Publication

Does not need a publication consent

Author contribution

Idea, concept and design: ES, SÇ, MÇ, EU

Data collection and analysis: ES, AGE, SÇ, MÇ, MO, EU,

Drafting of the manuscript: ES, AGE, EU, SÇ, MO

Critical review: ES, SÇ, MO, EU,

Data Availability

The data used to prepare this manuscript are available from the corresponding author when requested. Acknowledgements

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REFERENCES

1. Abbasi, N. A., Arukwe, A., Jaspers, V. L. B., Eulaers, I., Mennilod, E., Ibor, O. R., Frantz, A., Covaci, A., & Malik, R. N. (2017). Oxidative stress responses in relationship to persistent organic pollutant levels in feathers and blood of two predatory bird species from Pakistan. Science of the Total Environment, 580, 26-33. http://dx.doi.org/10.1016/j.scitotenv.2016.11.197

2. Acke, E., Midwinter, A. C., Lawrence, K., Gordon, S. J. G., Moore, S., Rasiah, I., Steward, K., French, N., & Walker, A. (2015). Prevalence of *Streptococcus dysgalactiae* subsp. *equisimilis* and *S. equi* subsp. *zooepidemicus* in a sample of healthy dogs cats and horses. New Zealand Veterinary Journal, 63, 265-271. http://dx.doi.org/10.1080/00480169.2015.1016133

3. Allam, N. M., & Lemcke, R. M. (1975). Mycoplasmas isolated from the respiratory tract of horses. The Journal of Hygiene, 74, 385-407. http://dx.doi.org/10.1017/s0022172400046908

4. Arnold, K. E., Larcombe, S. D., Ducaroir, L., & Alexander, L. (2010). Antioxidant status, flight performance and sexual signalling in wild-type parrots. Behavioral Ecology and Sociobiology, 64, 1857-1866. http://dx.doi.org/10.1007/s00265-010-0997-x

5. Black, P. A., David, D. V. M., & Horne, L. A. (2011). Hematologic parameters in raptor species in a rehabilitation setting before release. Journal of Avian Medicine and Surgery, 25(3), 192-198. https://doi.org/10.2307/41318139

6. Bize, P., Cotting, S., Devevey, G., Rooyen, J., Lalubin, F., Glaizot, O., & Christe, P. (2014). Senescence in cell oxidative status in two bird species with contrasting life expectancy. Oecologia, 174, 1097-1105. http://dx.doi.org/10.1007/s00442-013-2840-3

7. Campbell, T. W. (2012). Hematology of birds. In M. A. Thrall, G. Weiser, R. W. Allison & T. W. Campbell (Eds.), Veterinary hematology and clinical chemistry (2nd ed., pp 238-276). Wiley-Blackwell.

8. Casagrande, S., Dell'Omo, G., Costantini, D., Tagliavini, J., & Groothuis, T. (2011). Variation of a carotenoid-based trait in relation to oxidative stress and endocrine status during the breeding season in the Eurasian kestrel: A multi-factorial study. Comparative Biochemistry and Physiology Part A, 160, 16-26. http://dx.doi.org/10.1016/j.cbpa.2011.04.011.

9. Chaplin, S. B., Mueller, L. R., Degeneres, L. A. (1993). Physiological assessment of rehabilitated raptors prior to release. In P. T. Redig, J. E. Cooper, J. D. Remple, & D. B. Hunter (Eds.), Raptor Bio-medicine (pp 167-173). University of Minnesota Press.

10. Cohen, A. A., Hau, M., & Wikelski, M. (2008). Stress, metabolism, and antioxidants in two wild passerine bird species. Physiological and Biochemical Zoology, 81(4), 463-472. http://dx.doi.org/ 10.1086/589548

11. Coles, B. (2007). in: Essentials of Avian Medicine and Surgery, Nursing and after care, Ed. by B.H. Coles, (3rd ed.). Blackwell Publishing Ltd.

12. Cooper, J. E. (1972). Some haematological data for birds of prey. Raptor Research, 6, 133-136.

13. Costantini, D., & Dell'Omo, G. (2006). Environmental and genetic components of oxidative stress in wild kestrel nestlings (*Falco tinnunculus*). Journal of Comparative Physiology B, 176, 575-579. http://dx.doi.org/10.1007/s00360-006-0080-0

14. Costantini, D., Casagrande, S., Filippis, S., Brambilla, G., Fanfani, A., Tagliavini, J., & Dell'Omo, G. (2006). Correlates of oxidative stress in wild kestrel nestlings (*Falco tinnunculus*). Journal of Comparative Physiology B, 176, 329-337. http://dx.doi.org/10.1007/s00360-005-0055-6

15. Costantini, D., Coluzza, C., Fanfani, A., & Dell'Omo, G. (2007). Effects of carotenoid supplementation on colour expression, oxidative stress and body mass in rehabilitated captive adult kestrels (*Falco tinnunculus*). Journal of Comparative Physiology B, 177, 723-731. http://dx.doi.org/ 10.1007/s00360-007-0169-0

16. Costantini, D., & Verhulst, S. (2009). Does high antioxidant capacity indicate low oxidative stress? Functional Ecology, 23, 506–509. http://dx.doi.org/10.1111/j.1365-2435.2009.01546.x

17. Cram, D. L., Blount, J. D., York, J. E., & Young, A. J. (2015). Immune response in a wild bird is predicted by oxidative status, but does not cause oxidative stress. PlosOne, March: 1-10. https://doi.org/10.1371/journal.pone.0122421

18. Desmarchelier, M., Santamaria-Bouvier, A., Fitzgérald, G., & Lai, S. (2010). Mortality and morbidity associated with gunshot in raptorial birds from the province of Quebec: 1986 to 2007. Canadian Veterinary Journal, 51,70-74.

19. Espín, S., Martínez-López, E., Jiménez, P., María-Mojica, P., & García-Fernández, A. J. (2014). Effects of heavy metals on biomarkers for oxidative stress in Griffon vulture (*Gyps fulvus*). Environmental Research, 129, 59-68. http://dx.doi.org/10.1016/j.envres.2013.11.008

20. Fernie, K. J., & Bird, D. M. (2001). Evidence of oxidative stress in American Kestrels exposed to electromagnetic fields. Environmental Research, Section A, 86, 198-207. https://doi.org/10.1006/enrs.2001.4263

21. Fernie, K. J., & Reynolds, S. J. (2005). The effects of electromagnetic fields from power lines on avian reproductive biology and physiology: a review. Journal of Toxicology and Environmental Health, 8(2), 127-140, https://doi.org/10.1080/10937400590909022

22. Finkel, T., & Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. Nature, 408, 239-247. https://doi.org/ 10.1038/35041687

23. Fischer C. P., & Romero L. M. (2018). Chronic capti-

vity stress in wild animals is highly species-specific. Conservation Physiology, 7, 1-38. https://doi.org/10.1093/conphys/coz093

24. Fix, A. S., & Barrows, S. Z. (1990). Raptors rehabilitted in Iowa during 1986 and 1987: respospective study. Journal of Wildlife Diseases, 26(1), 18-21. https://doi.org/10.7589/0090-3558-26.1.18

25. Malik, A., & Valentine, A. (2018). Pain in birds: a review for veterinary nurses. Veterinary Nursing Journal, 33(1), 11-25. https://doi.org/10.1080/17415349.2017.1395304

26. Mander, C., Adams, L., & Riley, A. (2003). Wild City Neighbours: a guide to native bird rehabilitation. New Zealand Department of Conservation.

27. Mis, L., Mert, H., Comba, A., Comba, B., Söğütlü, İ. D., Irak, K., & Mert, N. (2018). Some mineral substance, oxidative stress and total antioxidant levels in Norduz and Morkaraman sheep. Van Veterinary Journal, 29(3), 131-134.

28. Morgan, K. N., & Tromborg, C. T. (2007). Sources of stress in captivity. Applied Animal Behaviour Science, 102, 262-302. https://doi.org/10.1016/j.applanim.2006.05.032

29. Redig, P. T. (1978). Raptor rehabilitation: diagnosis, prognosis and moral issues. In T. A. Geer (Ed.). Bird of prey management techniques (pp 29-41). British Falconer's Club.

30. Şahin, Ö. K., Aksoy, M. Ç., Uz, E., & Dağdeviren, B. H. (2015). Investigation of the effects of resveratrol on total oxidant/antioxidant capacity on experimental cigarette smoking model. SDÜ Sağlık Bilimleri Dergisi, 6(1), 10-14.

31. Vágási, C. I., Vincze, O., Pătraş , L., Osváth, G., Pénzes, J., Haussmann, M. F., Barta, Z., & Pap, P. L. (2019). Longevity and life history coevolve with oxidative stress in birds. Functional Ecology, 33, 152-161. https://doi.org/10.1111/1365-2435.13228

32. Wang, C., Zhao, F., Li, Z., Jin, X., Chen, X., Geng, Z., Hu, H., & Zhang, C. (2021). Effects of resveratrol on growth performance, intestinal development, and antioxidant status of broilers under heat stress. Animals, 11(1427), 2-10. https://doi.org/10.3390/ani11051427

33. Yaprakci, M. V., Ciğerci, İ. H., Ali, M. M., & Kabu, M. (2016). DNA damage, total antioxidant and oxidant status in gunshot wounded Wild Falcons. Pakistan Journal of Zoology, 48(5), 1417-1421.

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Research Article

Effect of vitamin C on the immune system in cattle immunized with blackleg vaccine

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ABSTRACT

The purpose of this study is to determine the effect of vitamin C on the immune system in cattle immunized against blackleg. The study employed 28 cattle of different breeds and genders, aged 15-20 months, bred in the Ardahan region. The cattle were divided into four groups, seven in each group. The first group (control group) 2 ml saline, the second group (vitamin group) 5 mg/kg vitamin c, the third group (vaccine group) 2 ml blackleg vaccine, and the fourth group (vaccine-vitamin group) 2 ml Blackleg vaccine and 5 mg/kg vitamin c was administered subcutaneously. Blood samples were collected from all animals in the groups just prior to vaccination and vitamin administration (day zero) and on the 14th and 28th days following administration. The number of formula leukocytes and the amount of serum IgG was measured in the blood samples. Serum IgG was found to increase substantially ($p<0.05$) in the vitamin-vaccine group on days 14 and 28 compared to the vaccine group. It was determined that the number of lymphocytes in days 14 and 28 of the vaccine-vitamin group increased in comparison with the vaccine group. In this study, it was found that vitamin c administered to cattle with the blackleg vaccine has a stimulating effect on the immune system. It is recommended to improve the protective potency of the vaccine using vitamin c along with the blackleg vaccine in cattle.

INTRODUCTION

Blackleg disease is a bacterial disease caused by *Clostridium chauvoei* (*Cl. Chauvoei*). The agent responsible for the disease is gram-positive, anaerobic and spored. These spores protect the bacteria against adverse environmental conditions. Because of spores, bacteria stay alive in the soil for a long time and maintain their ability to cause disease. In animal species, cattle are the most vulnerable to illness. It mainly affects cattle 6 to 30 months old. The disease progresses quickly and causes cattle to die (Abreu et al., 2017; Nicholson et al., 2019). The disease is also reported to be present in sheep and other animal species other than cattle (Abreu et al., 2017).

The disease has quite a complex pathogenesis. Spores ingested orally are carried from the digestive tract to muscle tissue through macrophages. These spores, which are latent in muscle tissue, change to a vegetative form when damaged. Some toxins are synthesized by bacteria that go through the vegetative form (Hansford, 2020). These toxins are responsible for muscle tissue inflammation and death (Abreu et al., 2017; Nicholson et al., 2019). Hyaluronidase, DNase, haemolysin, neuraminidase and leukocyte toxins play an important role in the pathogenesis of the disease. Moreover, it is suggested that the bacterial structural flagella are also effective in pathogenicity (Nicholson et al., 2019).

The disease begins in the muscle tissue. The affected muscular tissue is oedematous. In the future, the gas builds up and the cracking is felt. The infected animals have high fever. Anorexia and stagnation attract interest. The affected muscle tissue has a dark red or black appearance. Furthermore, fibrous areas and bleeding can be observed in the heart. Mortality is high in the illness (Abreu et al., 2017).

Vitamin C, also called ascorbic acid, is a water-soluble vitamin with important biological functions. In cattle, it is synthesized from d-glucose or d-galactose by glucuronic acid in the liver. Vitamin C is a co-factor in certain enzymes. It is required for the synthesis of collagen, catecholamines, peptide hormones, vasopressin, carnitine, cholesterol and tyrosine (Mousavi et al., 2019; Pogge & Hansen, 2013; Ranjan et al., 2012). Vitamin C deficiency leads to a disease known as scurvy (Matsui, 2012). Scurvy is characterized by a weakness in the structure of collagen. It has been reported that wound healing is delayed and susceptibility to life-threatening conditions such as pneumonia increases in patients with scurvy (Carr & Maggini, 2017; Mousavi et al., 2019). Vitamin C is decomposed by ruminal microflora in cattle and is not used orally. It is used parenterally and the dose is one to two grams (Kaya, 1997).

The best way to prevent blackleg disease is to immunize healthy animals. Vaccines are used for disease control around the world (Nicholson et al., 2019; Santos et al., 2021). In this country, cattle over four months old are vaccinated against the disease before they go to pasture. The vaccine is given behind the shoulder and subcutaneously (Mamak et al., 2018).

In a human study, vitamin C is reported to accumulate in neutrophils and monocytes (Carr & Maggini, 2017). There is also evidence that vitamin C increases serum antibody levels in humans (Mousavi et al., 2019). Based on this information, we can affirm that there is an important link between vitamin C and the immune system. This study was conducted to

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determine the effect of vitamin C given to cattle with the blackleg vaccine on the immune system.

MATERIAL and METHODS

This study has been approved by the Ethics Committee of Kafkas University (decision date 26.04.2022 and numbered 2022-085) and Ministry of Agriculture and Forestry of Turkey (letter dated 05.04.2022 and numbered E-5125179).

This research was carried out on 28 cattle of different breeds and genders, aged 15-20 months, in the Ardahan region. Cattle kept in the same environment were fed tap water from the same source and grassy grass. A dose of 0.2 mg/kg ivermectin (Vilmectin-Vilsan®) was administered subcutaneously to all cattle in the study of anti-parasitic purposes. There was one month of waiting period after the administration. Following this, the cattle were divided into four groups, seven in each group. 2 ml of saline (Polifleks®-Polifarma) to the first group (control group), 5 mg/kg of vitamin C (Maxivit-C®-baVET) to the second group (vitamin group), 2 ml of blackleg vaccine (VBR CHAUVOEI®-Ata-Fen) to the third group (vaccine group), and 5 mg/kg of vitamin C and 2 ml of blackleg vaccine to the fourth group (vaccine-vitamin group) were administered subcutaneously from different regions. Just before drug and vaccine administration (day zero) and on day 14 and 28 following administration, 2 ml of blood from all animals in the group into tubes with anticoagulant (EDTA) (BD Vacutainer® K2E 5.4 mg) and 10 ml of blood into tubes without anticoagulant (BD Vacutainer® CAT) samples were collected. The blood samples were taken to the laboratory in cold temperatures. Non-anticoagulant tubes were maintained at room temperature for 2 hours. Serums were then obtained by centrifuging at 3000 rpm for 20 minutes. The resultant serum samples were placed into an Eppendorf tube (İSOLAB®) and stored at -20^0 C until analysis. Blood smears were performed from blood samples in tubing containing anticoagulant. The leukocyte formula was determined using the conventional method (Yaman, 2016).

The ELISA kit (Bioassay Technology Laboratory, Cat. No: E0010Bo) was used to determine the quantity of G immunoglobulin (IgG) in serum samples. The analysis was carried out using the methodology described by the manufacturer. After the test, the optical density of the standard and serum samples was measured at 450nm in an ELISA reader (BioTek ELx800, U.S.A). Serum IgG was calculated by comparing the optical density of the samples to the optical density of the standards. This calculation was done by computer and using Microsoft Excel (Aydin, 2015).

The Microsoft Windows, SPSS 20.0 software program was used to statistically calculate the data obtained in the study. Normal distribution conditions were verified using the Shapiro-Wilk test. Group means were compared with one-way analysis of variance (ANOVA), multiple comparisons among groups were made using the Tukey HSD test. The results are presented on average and as a standard deviation. P<0.05 was found to be statistically significant in this study.

RESULTS

The serum IgG measured over 0, 14 and 28 days in cattle groups is presented in Table 1 below.

Table 1. Levels of serum IgG in the cattle groups (µg/ml)

Days	Control	Vitamin	Vaccine	Vaccine-vitamin
Day 0	54.60 ± 6.68	58.17 ± 17.89	57.14 ± 26.23	58.90 ± 29.68
Day 14	50.00 ± 22.40 ^{a}	49.54 ± 11.06 ^{a}	91.04 ± 13.13 ^{b}	116.58 ± 14.64 ^{c}
Day 28	48.55± 9.58 ^{a}	45.98± 17.85 ^{a}	97.31± 16.43 ^{b}	125.19± 14.07 ^{c}

^{a,b,c}: Those that have different letters in the same line in the P < 0.05 range were statistically significant.

Table 1 above shows that there is no difference in the amount of serum IgG on day zero among the groups. Serum IgG in the vaccination group at day 14 was found to have increased significantly (P<0.05) compared to the control and vitamin groups. In addition, the amount of IgG in the vaccination-vitamin group increased compared to the vaccinated group. It was observed that this significant increase in the amount of antibodies in the vaccine-vitamin group compared with the vaccine group continued on day 28.

Table 2 above demonstrates that there is no difference among the groups in terms of the percentage of leukocytes on day zero. The number of lymphocytes in the vaccine and vitamin-vaccine groups was found to increase significantly (P < 0.05) on day 14 compared to the control and vitamin groups. Lymphocytes in the vitamin-vaccine group were observed to increase on days 14 and 28 compared to the vaccine group. However, it was not statistically significant (P > 0.05).

DISCUSSION

Blackleg is a rapidly developing bacterial disease that kills cattle. It causes significant economic losses in the cattle breeding. Vaccines are used to fight the disease in this country and in countries around the world (Mamak et al., 2018; Nicholson et al., 2019; Santos et al., 2021). The synthesis of antibodies in a short period of time and at a protective level with vaccination is of great importance in the control of the disease. It is known that the development of immunity is possible with the stimulation of cells of the immune system. Vitamin C has been reported to have a significant relationship with the immune system. There is some evidence that plasma levels of vitamin C are declining in infectious diseases such as fatty liver disease, liver disease, heat stress, mastitis in cattle (Matsui, 2012; Ranjan et al., 2012).

There has been some suggestion that vitamin C is effective

Table 2. Leukocyte formula of the cattle groups by days (number/100)

Days	Parameters	Control	Vitamin	Vaccine	Vaccine- vitamin
Day 0	Neutrophil	27.71 ± 4.88	27.28 ± 4.30	27.85 ± 4.87	28.57 ± 2.82
	Eosinophil	12.71 ± 2.87	11.28 ± 2.87	10.42 ± 2.63	11.42 ± 2.99
	Basophil	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Monocyte	1.28 ± 1.11	1.14 ± 1.21	1.14 ± 1.06	1.00 ± 1.15
	Lymphocyte	55.14 ± 6.71	54.28 ± 5.85	54.71 ± 4.82	57.28 ± 5.25
Day 14	Neutrophil	26.28 ± 5.05	26.57 ± 5.15	28.57 ± 5.34	29.71 ± 1.79
	Eosinophil	11.28 ± 2.92	10.85 ± 2.03	10.57 ± 2.37	10.71 ± 2.49
	Basophil	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Monocyte	1.14 ± 1.21	1.00 ± 1.15	1.28 ± 1.38	1.42 ± 1.61
	Lymphocyte	54.71 ± 7.49 [a]	55.71 ± 4.85 [a]	62.42 ± 3.86 [b]	65.57 ± 3.95 [b]
Day 28	Neutrophil	27.28 ± 3.81	25.42 ± 4.42	26.71 ± 4.46	28.28 ± 2.87
	Eosinophil	11.85 ± 2.11	11.28 ± 2.13	10.57 ± 2.82	11.57 ± 2.37
	Basophil	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Monocyte	1.00 ± 1.15	1.14 ± 1.21	0.71 ± 0.75	1.14 ± 1.21
	Lymphocyte	53.85 ± 4.37 [a]	54.57 ± 3.50 [a]	60.14 ± 4.94 [b]	64.57 ± 4.72 [b]

[a,b]: Those that have different letters in the same line in the $P < 0.05$ range were statistically significant.

in increasing disease resistance in cattle. Vitamin C use in calves is reported to reduce diarrhea (Cummins & Brunner, 1989; Sahinduran & Albay, 2004; Seifi et al., 1996). It has been observed that when applied to cattle prior to transport, vitamin C prevents the reduction in plasma ascorbate due to transport and increases fattening performance (Deters & Hansen, 2020).

Many studies have demonstrated the association between vitamin C and antibody levels in cattle. It has been reported that when the calves received colostrum are used orally 3 grams a day in the first week, 2 grams in the second week, and 1 gram in the third week, the amount of serum gamma globulin is increased significantly on the 14th day but not significantly on the 28th day (Lotfollahzadeh et al., 2005). In a study, vitamin C administered to calves was found to not affect the amount of total plasma IgG (Hidiroglou et al., 1995). In another study, calves were given 1 g of vitamin C per day from 2 weeks to 8 weeks of age, and the same calves were vaccinated with inactivated *Histophilus somni* vaccine when they reached 4 and 8 weeks of age. It is reported that 12 weeks after the second vaccination, vitamin C greatly increased the amount of antibodies synthesized against *Histophilus somni* (Otomaru et al., 2021). When vitamin C is administered to cattle on the 3rd day of foot-and-mouth vaccination, it leads to an increase in serum gamma globules (Kızıl & Gül, 2010). Another study suggests that vitamin C accelerates treatment and increases the amount of milk immunoglobulin in dairy cattle suffering from mastitis (Chaiyotwittayakun et al., 2002).

The effect of vitamin C on the immune system has been studied in some animal species besides cattle. It was suggested that 200 mg/kg of vitamin C administered intramuscularly to sheep prior to transplant is effective in preventing stress from transport (Yanar, 2020). When the goats were 21 days old, they

were separated from their mothers and exposed to stress and given 100 mg of vitamin C orally per day. The same goats were vaccinated against *Brucella melitensis* at 14 weeks old. It was reported that there was no significant increase in serum antibody levels in the vitamin C group at three weeks post-vaccination (İmik et al., 2000).

The effect of vitamin C on the immune system has also been studied in poultry. It is claimed that ascorbic acid added to the diets of chickens exposed to stress by applying heat for 35 days increases the growth performance and the amount of antibodies against Newcastle virus (Gouda et al., 2020). In another study, chickens raised in an overcrowded environment were supplemented with vitamin C in their diet and immunized against the Newcastle virus. It is reported that the quantity of antibodies synthesized against the vaccine does not increase significantly 7 and 14 days after vaccination (Mirfendereski & Jahanian, 2015). In another study, chickens were vaccinated against Newcastle and infectious bronchitis after being molted with zinc oxide added to their feed. After vaccination, the level of antibodies in the group to which vitamin C was added increased insignificant relative to the control group (Khan et al., 2014). In another study, it was reported that daily supplementation of 500 mg/kg ascorbic acid to broilers exposed to heat stress from 22 days to 42 days increased the amount of antibodies against Newcastle disease and reduced the negative effects of heat (Toplu et al., 2014). In another study, the amount of antibodies against Infectious Bursal Disease was found to be higher in chickens supplemented with ascorbic acid in their diets than in the group that did not receive ascorbic acid (Wu et al., 2000). It has been reported that vitamin C added to the feed laying hens has no effect on the antibody titer against Newcastle disease (Coşkun et al., 1998). It is suggested that vitamin C injected at a dose of 3 mg on the 15th day increases the hatchability rate, the amount of IgG and IgM (Zhu et al., 2019).

Human studies have suggested that there is a positive relationship between vitamin C and antibody levels (IgA, IgG, IgM) (Carr & Maggini, 2017). Enhanced antibody production (IgG and IgM) was observed in human lymphocytes exposed to vitamin C in vitro (Tanaka et al., 1994). It has been reported that intra-peritoneal administration of vitamin C in guinea pigs increases mitotic activity and levels of post-vaccination antibodies in lymphocytes (Carr & Maggini, 2017). The FMD inactivated intramuscular vaccine was administered 7 and 14 days after 1 mg and 10 mg intra-peritoneal injection of vitamin C to the rats divided into groups. The high dose group for vitamin C was reported to show a significant increase in serum IgG (Wu et al., 2018).

In the present study, it was found that there was no difference between serum IgG in the control groups and vitamins in days 0, 14 and 28. The amount of IgG measured on days 14 and 28 in the vaccine-vitamin group was found to have increased significantly ($P<0.05$) compared to the vaccine group. It has been reported that vitamin C increases the amount of serum antibody in newborn calves (Lotfollahzadeh et al., 2005), calves vaccinated against *Histophilus somni* (Otomaru et al., 2021), in beef cattle vaccinated against Foot and Mouth disease (Kızıl & Gül, 2010), in dairy cattle with mastitis (Chaiyotwittayakun et al., 2002), in goats vaccinated with *Brucella melitensis* (İmik et al., 2000), chickens vaccinated against Newcastle virus (Gouda et al., 2020; Khan et al., 2014; Mirfendereski & Jahanian, 2015; Toplu et al., 2014), in chickens vaccinated against Infectious Bronchitis (Khan et al., 2014), in chickens vaccinated against Infectious Bursal Disease (Wu et al., 2000), when injected into embryonated eggs (Zhu et al., 2019), in human lymphocytes in vitro (Tanaka et al., 1994), in mice vaccinated with Foot and Mount Disease (Wu et al., 2018). The results obtained in this study are consistent with the research results presented above. Serum IgG levels in the vaccine-vitamin group showed a significant increase in comparison to the vaccine group. This increase may be because of vitamin C stimulating the mitotic activity of lymphocytes. It may be interpreted that lymphocytes with increased mitotic activity differ into antibody synthesis plasma cells and cause increased antibody production.

Contrary to the findings of this study, it is reported that vitamin C does not affect the level of antibody in calves (Hidiroglou et al., 1995), and in chicken vaccinated with Newcastle vaccine (Coşkun et al., 1998). In some studies, it has been suggested that vitamin C supplementation decreases the incidence of diarrhea in calves (Cummins & Brunner, 1989; Sahinduran & Albay, 2004; Seifi et al., 1996), improves the fattening performance of cattle (Deters & Hansen, 2020), and sheep (Yanar, 2020). It is thought that the stress factors that animals are exposed to may be effective among the reasons for obtaining different results between vitamin C administration and the amount of antibodies.

In this study, it was found that there was no difference in the number of leukocytes in the control and vitamin groups during the 0, 14 and 28 days. It is observed that the lymphocyte counts of the vaccine and vaccine-vitamin group increased significantly ($P < 0.05$) on the 14th and 28th days compared to the control and vitamin groups. It was observed that the lymphocyte counts of the vaccine-vitamin group increased on the 14th and 28th days compared to the vaccine group. However, the increase was statistically insignificant ($P > 0.05$). No differences were found between the neutrophil, eosinophil, basophil and monocyte counts in the 0th, 14th and 28th day groups. One study indicates that lymphocytes have 10 to 100 times more vitamin C than plasma (Mousavi et al., 2019). It has been suggested that intra-peritoneal administration of vitamin C to guinea pigs increases the mitotic activity of lymphocytes (Carr & Maggini, 2017). In another study, it was reported that vitamin C administered at different doses (25mg, 50mg and 75mg/kg/day for 30 days) to sheep exposed to heat stress increased the lymphocyte count (Babe, 2011). The results of this research are similar to those of Babe (2011), Carr and Maggini (2017), and Mousavi et al. (2019). The reason for this increase in the number of lymphocytes may be due to the accumulation of vitamin C in lymphocytes and stimulating lymphocytes in the direction of maturation, differentiation and proliferation.

CONCLUSION

Blackleg is a rapidly developing bacterial disease that kills cattle. It causes significant economic losses in the cattle

breeding. The best way to prevent blackleg disease is to immunize healthy animals. In this study, it was observed that vitamin C, injected with 5mg/kg in different regions together with the Blackleg vaccine, stimulated the immune system by increasing the amount of serum IgG and lymphocyte count. Because of this immune-boosting effect, it is recommended to use vitamin C along with the blackleg vaccine in cattle. Thus, the protective effect of the vaccine may be increased against acute and fatal blackleg disease, which does not have a cure.

DECLARATIONS

Ethics Approval

This study has been approved by the Ethics Committee of Kafkas University (decision date 26.04.2022 and numbered 2022-085) and Ministry of Agriculture and Forestry of Turkey (letter dated 05.04.2022 and numbered E-5125179).

Conflict o Interest

The author declares that there is no conflict of interest.

Author contributions

All applications were performed by the author.

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Data availability

The data used to prepare this manuscript are available from the corresponding author when requested.

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Not applicable.

REFERENCES

1. Abreu, C. C., Edwards, E. E., Edwards, J. F., Gibbons, P. M., Leal de Araújo, J., Rech, R. R., & Uzal, F. A. (2017). Blackleg in cattle: A case report of fetal infection and a literature review. Journal of Veterinary Diagnostic Investigation, 29(5), 612–621. https://doi.org/10.1177/1040638717713796

2. Aydin, S. (2015). A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA. Peptides, 72(1), 4–15. https://doi.org/10.1016/j.peptides.2015.04.012

3. Babe, A. A. (2011). Effect of vitamin C on haematology and serum biochemistry in heat-stressed sheep. Research Opinions in Animal & Veterinary Sciences, 1(11), 731–733.

4. Carr, A. C., & Maggini, S. (2017). Vitamin C and immune function. Nutrients, 9(11), 1–25. https://doi.org/10.3390/nu9111211

5. Chaiyotwittayakun, A., Erskine, R. J., Bartlett, P. C., Herdt, T. H., Sears, P. M., & Harmon, R. J. (2002). The effect of ascorbic acid and L-histidine therapy on acute mammary inflammation in dairy cattle. Journal of Dairy Science, 85(1), 60–67. https://doi.org/10.3168/jds.S0022-0302(02)74053-8

6. Coşkun, B., Inal, F., Erganiş, O., Kuyucuo□lu, Y., Ok, U., Çelik, I., Tiftik, A. M., & Kurto□lu, F. (1998). Farklı Düzeylerde Askorbik Asit İhtiva Eden Rasyonların Yumurta Tavuklarında Verim ve İmmünite Üzerine Etkileri. Turkish Journal of Veterinary and Animal Sciences, 22(1), 29–35.

7. Cummins, K. A., & Brunner, C. J. (1989). Dietary Ascorbic Acid and Immune Response in Dairy Calves. Journal of Dairy Science, 72(1), 129–134. https://doi.org/10.3168/jds.S0022-0302(89)79088-3

8. Deters, E. L., & Hansen, S. L. (2020). Pre-transit vitamin C injection improves post-transit performance of beef steers. Animal, 14(10), 2083–2090. https://doi.org/10.1017/S1751731120000968

9. Gouda, A., Amer, S. A., Gabr, S., & Tolba, S. A. (2020). Effect of dietary supplemental ascorbic acid and folic acid on the growth performance, redox status, and immune status of broiler chickens under heat stress. Tropical Animal Health and Production, 52(6), 2987–2996. https://doi.org/10.1007/s11250-020-02316-4

10. Hansford, J. (2020). Blackleg without skeletal muscle involvement as a cause of sudden death in unvaccinated calves. Canadian Veterinary Journal, 61(1), 189–192.

11. Hidiroglou, M., Batra, T. R., Ivan, M., & Markham, F. (1995). Effects of Supplemental Vitamins E and C on the Immune Responses of Calves. Journal of Dairy Science, 78(7), 1578–1583. https://doi.org/10.3168/jds.S0022-0302(95)76781-9

12. İmik, H., Aytaç, M., Coşkun, B., & Fidancı, H. (2000). Strese Maruz Bırakılan Ankara Keçisi Oğlaklarında E ve C Vitaminlerinin Büyüme ve İmmünite Üzerine Etkileri. Turkish Journal of Veterinary and Animal Sciences, 24(1), 51–58.

13. Kaya, S. (1997). Beslenme Farmakolojisi. In S. Kaya, İ. Pirinçci, & A. Bilgili (Eds.), Veteriner Uygulamalı Farmakoloji (1.Baskı, pp. 232–234). Medisan Yayınevi.

14. Khan, R. U., Rahman, Z. U., Javed, I., & Muhammad, F. (2014). Effect of vitamins, protein level and probiotics on immune response of moulted male broiler breeders. Journal of Animal Physiology and Animal Nutrition, 98(4), 620–627. https://doi.org/10.1111/jpn.12087

15. Kızıl, Ö., & Gül, Y. (2010). Şap Aşısı Uygulanan Besi Sığırlarında AD3E ve C Vitamini Uygulamalarının Serum Protein Fraksiyonları Üzerine EtkileÜniverri. Fırat Üniversitesi Sağlık Biimleri Veteriner Dergisi, 24(2), 57–62.

16. Lotfollahzadeh, S., Mokhber Dezfouli, M. R., Khazraii Nia, P., & Tajik, P. (2005). The effects of oral administration of ascorbic acid on serum γ-globulin concentration of neonatal calves. Iranian Journal of Veterinary Research, 6(2), 79–83.

17. Mamak, N., Kale, M., & Yapıcı, O. (2018). Sığır Yetiştiriciliğinde Kullanılan Aşılar ve Önemi. Göller Bölgesi Aylık Hakemli Ekonomi ve Kültür Dergisi, 5(63), 61–66.

18. Matsui, T. (2012). Vitamin C nutrition in cattle. Asian-Australasian Journal of Animal Sciences, 25(5), 597–605. https://doi.org/10.5713/ajas.2012.r.01

19. Mirfendereski, E., & Jahanian, R. (2015). Effects of dietary organic chromium and vitamin C supplementation on performance, immune responses, blood metabolites, and stress status of laying hens subjected to high stocking density. Poultry Science, 94(2), 281–288. https://doi.org/10.3382/ps/peu074

20. Mousavi, S., Bereswill, S., & Heimesaat, M. M. (2019). Immunomodulatory and antimicrobial effects of vitamin C. European Journal of Microbiology and Immunology, 9(3), 73–79. https://doi.org/10.1556/1886.2019.00016

21. Nicholson, P., Furrer, J., Hässig, M., Strauss, C., Heller, M., Braga-Lagache, S., & Frey, J. (2019). Production of neutralizing antibodies against the secreted Clostridium chauvoei toxin A (CctA) upon blackleg vaccination. Anaerobe, 56, 78–87. https://doi.org/10.1016/j.anaerobe.2019.02.011

22. Otomaru, K., Oishi, S., Fujimura, Y., Iwamoto, Y., Nagai, K., & Ijiri, M. (2021). Effects of vitamin C supplementation on the blood oxidative stress and antibody titre against Histophilus somni vaccination in calves. Journal of Veterinary Research, 65(1), 73–78. https://doi.org/10.2478/jvetres-2021-0002

23. Pogge, D. J., & Hansen, S. L. (2013). Supplemental vitamin C improves marbling in feedlot cattle consuming high sulfur diets. Journal of Animal Science, 91(9), 4303–4314. https://doi.org/10.2527/jas.2012-5638

24. Ranjan, R., Ranjan, A., Dhaliwal, G. S., & Patra, R. C. (2012). L-Ascorbic acid (vitamin C) supplementation to optimize health and reproduction in cattle. Veterinary Quarterly, 32 (3–4), 145–150. https://doi.org/10.1080/01652176.2012.734640

25. Sahinduran, S., & Albay, M. K. (2004). Supplemental ascorbic acid and prevention of neonatal calf diarrhoea. Acta Veterinaria Brno, 73(2), 221–224. https://doi.org/10.2754/avb200473020221

26. Santos, F. D. S., Maubrigades, L. R., Gonçalves, V. S., Alves Ferreira, M. R., Brasil, C. L., Cunha, R. C., Conceição, F. R., & Leite, F. P. L. (2021). Immunomodulatory effect of short-term supplementation with Bacillus toyonensis BCT-7112T and Saccharomyces boulardii CNCM I-745 in sheep vaccinated with Clostridium chauvoei. Veterinary Immunology and Immunopathology, 237, 110272. https://doi.org/10.1016/j.vetimm.2021.110272

27. Seifi, H. A., Mokhber Dezfuly, M. R., & Bolurchi, M. (1996). The effectiveness of ascorbic acid in the prevention of calf neonatal diarrhoea. Journal of Veterinary Medicine, Series B, 43(3), 189–191. https://doi.org/10.1111/j.1439-0450.1996.tb00304.x

28. Tanaka, M., Muto, N., Gohda, E., & Yamamoto, I. (1994). Enhancement by Ascorbic Acid 2-Glucoside or Repeated Additions of Ascorbate of Mitogen-Induced IgM and IgG Productions by Human Peripheral Blood Lymphocytes. The Japanese Journal of Pharmacology, 66(4), 451–456. https://doi.org/10.1254/jjp.66.451

29. Toplu, H. D. O., Tunca, R., Aypak, S. U., Coven, F., Epikmen, E. T., Karaarslan, S., & Yagin, O. (2014). Effects of heat conditioning and dietary ascorbic acid supplementation on heat shock protein 70 expression, blood parameters and fear-related behavior in broilers subjected to heat stress. Acta Scientiae Veterinariae, 42(1), 1179.

30. Wu, C., Dorairajan, T., & Lin, T. (2000). Effect of ascorbic acid supplementation on the immune response of chickens vaccinated and challenged with infectious bursal disease virus. Veterinary Immunology and Immunopathology, 74 (1–2), 145–152. https://doi.org/10.1016/S0165-2427(00)00161-6

31. Wu, M., He, M., & Kang, Y. (2018). Vitamin C supplementation improved the efficacy of foot-and-mouth disease vaccine. Food and Agricultural Immunology, 29(1), 470–483. https://doi.org/10.1080/09540105.2017.1406459

32. Yaman, K. (2016). Fizyoloji (5. Baskı). Ezgi Kitabevi.

33. Yanar, K. E. (2020). Morkaraman Irkı Koyunlarda Uzun Süreli Transportta Oluşan Stresin Neden Olduğu İmmun Supresyon Üzerine Parapoxvirus Ovis, Corynebacterium Cutis Lizatı ve C vitamininin Etkisinin Araştırılması. Atatürk Üniversitesi Sağlık Bilimleri Enstitüsü, Doktora Tezi, Erzurum.

34. Zhu, Y. F., Li, S. Z., Sun, Q. Z., & Yang, X. J. (2019). Effect of in ovo feeding of Vitamin C on antioxidation and immune function of broiler chickens. Animal, 13(9), 1927–1933. https://doi.org/10.1017/S1751731118003531

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Research Article

Investigation of ectoparasites in budgerigar and canaries in Burdur city of Turkey

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ABSTRACT

Ectoparasite infestations are one of the most important but neglected diseases of birds. Ectoparasites cause sirious harmful effects such as; irritation, feathers and skin damage, restlessness, anemia, weight loss, transmission of other pathogens, reducing overall fitness and decreasing long term survival depending on the intensity of the infestation. This study was performed to investigate the ectoparasites of budgerigars and canaries in the center district of Burdur city, Turkey. For this purpose, we randomly selected 555 budgies from 192 cages and 121 canaries from 49 cages owned by 12 different breeders. The feathers of head, neck, abdomen, legs, tail, beak, under the wings, around the cloaca and feet of all birds, in addition cages and cage equipments were examined in terms of ectoparasite infestations. As a result; no ectoparasite species were found on/between feathers and other body parts of overall 676 cage birds, however, a large number of *Dermanyssus gallinae* were found in cages and cage equipments. *Dermanyssus gallinae* detected in rates of 28.65% and 28.57% in budgerigar and canary cages, respectively. The present study is the first investigation on ectoparasites of budgerigar and canaries in Turkey and rarely in the world. In addition, within this study, *D. gallinae* infestation was reported for the first time from budgerigar and canaries from Turkey.

INTRODUCTION

The interaction between human and birds dates back many thousands years ago when people began to use these animals for meat, eggs, leather, feathers, bones, hunting or other purposes and today some species of birds continue to be a part of families as 'pets' in many countries in the world (Anderson, 2010). The 'pet bird' term is being used for birds housed and fed particularly for ornamental use, which can be classified mainly in two groups; Passeriformes (canaries, finches, mynah, sparrows, etc.) called songbirds and Psittaciformes (budgerigars, parakeets, parrots, etc.) (Boseret et al., 2013; Fard et al., 2020). Health and welfare of ornamental birds is especially essential because these animals have close bonds with humans (Anderson, 2010; Fard et al., 2020).

Ectoparasite infestations are one of the most important and neglected (Moller et al., 1990) diseases of the birds. Ectoparasites cause sirious harmful effects such as; irritation, feathers and skin damage, restlessness, anemia, weight loss, transmission of other pathogens, reducing overall fitness (nestling survival, reproductive success etc.) and decreasing long term survival depending on the intensity of the infestation (Moller and Lope, 1999; Kehlmaier and Quassier, 2013; Fard et al., 2020). As far as is known; several species of Acarina (mites), Ixodida (ticks), Phthyraptera (lice), Heteroptera (bugs), Siphonaptera (fleas) and Diptera (flies) can be parasitized on birds (Kehlmaier and Quassier, 2013; Ombugadu et al., 2019).

Lice species seen on birds are included in the suborders Ischnocera and Amblycera of the Phthiraptera order and have chewing type of mouth structure, so they fed on skin debris and feathers. Because lices are obligate-permanent ectoparasites, they can not survive without a host for so long, therefore, the entire life cycle must be completed on the host (Ombugadu et al., 2019). Following lice, mites (Acari) considered the second richest ectoparasitic group parasitized on birds (Negm et al., 2018). Ectoparasitic mites of birds can be categorized mainly under two groups; those living in/around the nest and permanently living on the host (Proctor and Owens, 2000). Ixodida (ticks) and Mesostigmata suborders of mites include most of the temporary and permanent species (Proctor and Owens, 2000). Ticks fed by blood sucking for a while and leaving hosts, spend most of their time in hidden areas like nests, burrows, crevices and cracks. Several soft (Argasidae) and hard (Ixodidae) tick species parasitized on birds can lead to anemia and paralysis (Gothe and Neitz, 1991) and also transmit some pathogens such as bacteria, virus and protozoa (Shah et al., 2004). Another blood-sucking and temporary ectoparasitic mite group belongs to *Dermanyssus* and *Ornithonyssus* genera. These are generally known, as 'fowl mites' or 'red poultry mite', but actually their natural hosts are small passerine birds (Proctor and Owens, 2000). *Dermanyssus gallinae* fed by sucking blood, especially at night, from a very wide range of hosts such as chicken, pigeon, house sparrow, starling, pet canaries, parakeets, rarely cat, dog, rodent, horse and even humans (Mullen and Oconnor, 2002; Marchiondo and Endris, 2019).

According to literature review; there are several studies on ectoparasites of ornamental or cage birds in the world (Heylen and Mattyhssen, 2008; Kounek et al., 2011; Boseret et al., 2013; Kehlmaier and Quaisser, 2013; Moodi et al., 2013; Liv-

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inius et al., 2018; Saranya et al., 2018; Fard et al., 2020). However, there are limited studies on this subject in Turkey (Dik et al., 2011; 2013).

The aim of the present study was to investigate the ectoparasites of the ornamental birds (budgerigars, canaries) in Burdur city of Turkey.

MATERIAL and METHODS

Study area, animals and sampling

The study was conducted from September to December 2020 on randomly selected 555 budgies in 192 cages and 121 canaries in 49 cages belonging to 12 different breeders in the center district of Burdur city, Turkey. The feathers of the head, neck, abdomen, legs, tail, beak, under the wings, around the cloaca and feet of all birds also cage equipments were examined in terms of ectoparasites. Detected samples were collected into eppendorf tubes containing 70% ethanol and transferred to the laboratory.

Processing and identification of ectoparasite

The collected samples were cleared in 10% KOH about 3 to 5 days, washed in distilled water for 24 hours and dehydrated in 70%, 80%, 90% and 99% series of ethanol (one day for each), respectively, then the fixed slides were mounted with Canada balsam (Dik et al., 2013; Fard et al., 2020). Measurement of the body dimensions of collected arthropods performed using Olympus BX51 microscope and cellSense Standard 1.18 software program. The identification was carried out with the help of diagnostic key literature (Moss, 1968).

Statistical analysis

Statistical analysis of the data obtained in this study was performed with the help of MiniTab16 statistical software with the Pearson chi-square test. The chi-square test was used to compare the infestation rates between budgerigars and canaries, and the observed differences were considered statistically significant if the P value found was less than 0.05.

RESULTS

Depending on detailed examinations, no ectoparasite species were found on/between feathers and other body parts of overall 676 cage birds (555 budgeries, 121 canaries). However, a large number of *D. gallinae* was detected in cages and cage equipments. All developmental stages; 700 females, 247 males, 1142 proto and deutonymphs, 172 larvae and 1296 eggs of *D. gallinae* were identified as seen in Figure 1 and Figure 2.

Infestation rates with *D. gallinae* of budgerigar and canary cages presented in Table 1. Overall *D. gallinae* infestation rates

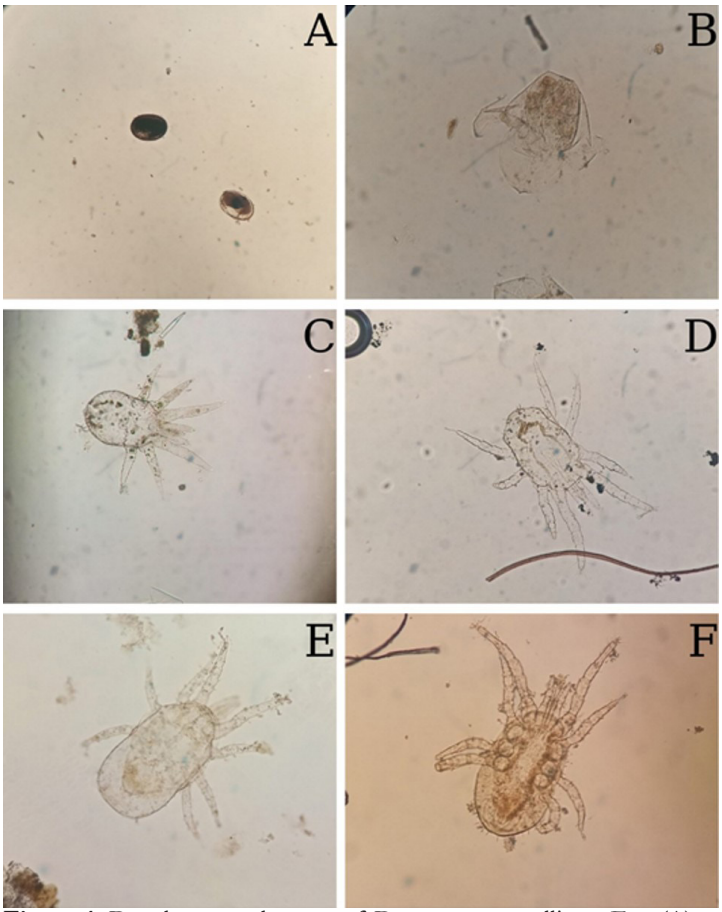

Figure 1. Developmental stages of Dermanyssus gallinae; Egg (A), hatching larva (B), larva (C), protonymf (D), deutonymf (E), male (F) (4x objective)

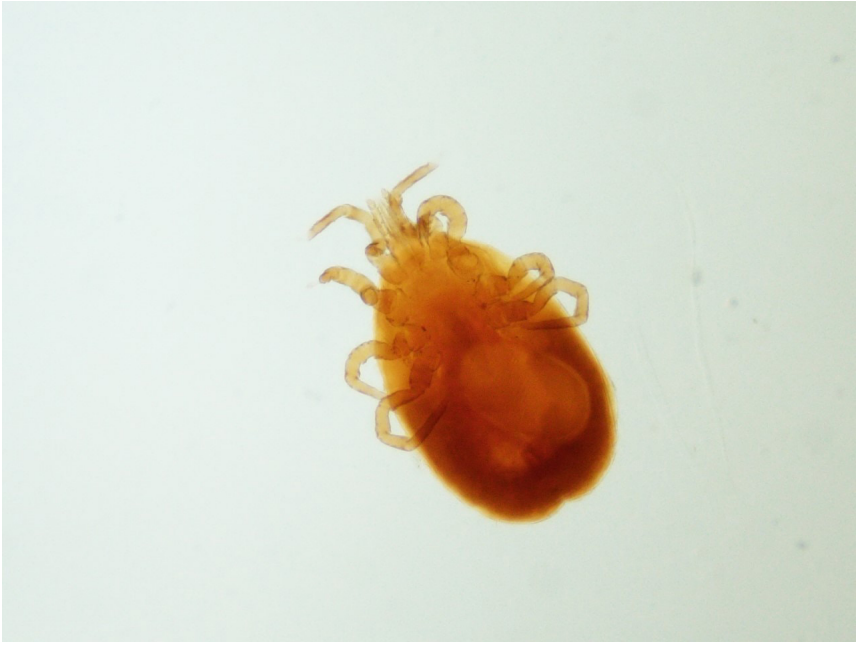

Figure 2. Female *Dermanyssus gallinae* with egg (4x objective)

Table 1. Number of non-infested and infested budgerigar and canary cages with *D. gallinae*

Breeder No	Number of budgie cage	Number of budgies	Number of infested budgie cage	Number of canary cage	Number of canaries	Number of infested canary cage
1	41	160	4	21	57	-
2	78	202	39	7	12	6
3	20	54	-	-	-	-
4	-	-	-	5	12	5
5	-	-	-	4	6	-
6	-	-	-	5	16	-
7	9	15	8	3	3	3
8	-	-	-	3	13	-
9	20	66	-	-	-	-
10	20	49	-	-	-	-
11	4	9	4	-	-	-
12	-	-	-	1	2	-
Total	**192**	**555**	**55**	**49**	**121**	**14**

were 41.67% (5/12) and 28.63% (69/241) for breeders and cages, respectively. For budgerigars; *D. gallinae* was detected in 4 of 7 breeders (57.14%), 55 of 192 cages (28.65%) and for canaries in 3 of 8 breeders (35.5%) and 14 of 49 cages (28.57%).

According to the data; no statistical significance was found for *D. gallinae* infestation rates between budgerigar and canary cages and equipments ($\chi 2 = 0.000$; DF = 1; P-Value = 0.992).

DISCUSSION

Ectoparasitic infestations should not be ignored, especially because of causing harmful effects on the survival, development and reproductive success of birds (Hamstra and Badyaev, 2009). In addition, the fact that ectoparasites are vectors for various viral, bacterial and parasitic pathogens increases their importance even more (Moller et al., 1990; Kehlmaier and Quassier, 2013; Fard et al., 2020).

In some studies in Turkey (Dik et al., 2011; 2013) wild birds were caught for a short time with various traps, the ectoparasites on them were collected, and then birds were released to natural habitats. There are case reports about ectoparasites of cage birds from Turkey (Dik, 2010; Koc et al., 2017) and some other countries (Sychra et al., 2007; Alarcon Elbal et al., 2014). In addition, there are some studies on ectoparasites detected on birds in zoos (Sychra, 2005; Prathipa et al., 2013; Tags et

al., 2020).

In a study conducted in the Czech Republic; three species of Ornithomya (louse-flies), two species of fleas (*Ceratophyllus* and *Dasypsyllus*), 15 species of chewing lice (Myrsidea, Menacanthus, Brueelia, Penenirmus, Philopterus) were reported on 82 birds of 23 wild passerine bird (Passeriformes) species (Sychra et al., 2008). In a study performed in Bangladesh (Musa et al, 2012); *Menopone gallinae* (16.66%), *Menacanthus starmineus* (33.33%), *Colpocephalum turbinatum* (33.33%), *Columbicola columbae* (50%) and *Lipeurus caponis* (25%) lice species were reported from 24 pigeons as given incidences. In a study from India (Prathipa et al, 2015); *Syringophilus* sp. (14.51%) and *Dermoglyphus* sp. (17.74%) infestations were reported on psittacine birds (budgerigars, African love birds and cockatiels). Cristofani et al. (2017) reported *Ornithonyssus sylviarum* and *Menacanthus eurysternus* infestations for the first time from Italy in 78 captive canaries from an aviary. In another similar study; Saranya et al., (2018) reported *Columbicola* sp., *Menocanthus* sp., *Pseudolynchia* sp. and *Anaticola* sp. infestations on captive wild birds from India. In a study conducted in Iran (Fard et al., 2020); *M. gallinae*, *M. stramineus*, *C. columbae*, *Goniodes pavonis*, *Myrsidea fasciata*, an unknown species from *Philopetrus* genus, *Argas reflexu* , Pseudolinchya, and Culicoides infestations were reported in 318 birds belonging to four orders of Psittacines, Columbiformes, Passeriformes, and poultry were examined for ectoparasites. However, no ectoparasite infestation was observed on examined canary and budgerigars in the same study (Fard et al., 2020). From Ankara, the capital city of Turkey, *D.* gallinae infestations were reported on two species of parrots in a case report study (Koç et al, 2017).

Best of our knowledge; the present study is the first investigation study of ectoparasite of budgerigar and canaries in Turkey and one of the few studies in the world. In addition, within this study, *D. gallinae* infestation is reported for the first time from budgerigar and canaries from Turkey. The fact that only *D. gallinae* was detected in this study does not mean that only this species is parasitizing as ectoparasite in cage birds in the Burdur region and Turkey. It is necessary to conduct detailed studies in different regions using various methods to widen our knowledge about the prevalence of ectoparasites and the epidemiology of the diseases they cause. Additionally, no statistical significance was found on the budgerigar and canary cages and equipments' infestation rates with *D. gallinae*. Based on these findings, it can be concluded that *D. gallinae* does not prefer one of these two bird species in terms of feeding and it has a similar tendency for both bird species.

As an obligate hematophagous ectoparasite, red mite *D. gallinae*, suck blood from mainly poultry, less common pet birds, and even humans. In addition to its direct parasitic effects such as anemia, weight loss, dermatitis and decrease in egg production (Arends, 2008), it also has importance as a carrier of several vectorborne pathogens (De luna et al., 2008; Sparagano et al., 2014; Sommer et al., 2016). Transmission of bacteria and viruses such as *Salmonella enteritidis*, *Pasteurella multocida*, *Coxiella burnetti*, Spirochetes, Fowl poxvirus, Eastern, Western and Venezuelan equine encephalitis viruses via *D. gallinae* to another host have been demonstrated. While some others (*S. gallinarum*, *Chlamydia* spp., *Escherichia coli*, *Staphylococcus* spp., *Streptomyces* spp., Newcastle disease virus, *Plasmodium* spp.) were just isolated from red mite and transmission not proven yet (Valiente Moro et al., 2009; Ciloglu et al., 2020). Due to its ability to suck blood from humans and its potential vector role to various pathogen agents for both humans and animals, it seems necessary to take preventive measures and carry out more studies about *D. gallinae* and other arthropod parasites of ornamental or cage birds.

CONCLUSION

In conclusion; this is the first investigation study of ectoparasites of budgerigar and canaries in Turkey. *D. gallinae* infestation is also reported for the first time from budgerigar and canaries from Turkey within this study. Further studies need to be performed to widen our knowledge about ectoparasite infestations of ornamental birds and vectorial potential of arthropods.

DECLARATIONS

Ethics Approval

The study was approved by the Animal Experiments Local Ethics Committee of Burdur Mehmet Akif Ersoy University (2020/694).

Conflict o Interest

The authors declare that they have no competing interests.

Consent for Publication

During the sample collection, informed consents had been obtained from all animal owners/breeders.

Author contribution

Idea, concept and design: ÖY, OK

Data collection and analysis: ÖY, OK

Drafting of the manuscript: ÖY, OK

Critical review: ÖY, OK

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding authors on reasonable request.

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REFERENCES

1. Alarcon Elbal, P. M., Carmona Salido, V. J., Sanchez Murillo, J. M., Calero Bernal, R. & Lucientes Curdi, J. (2014). Severe beak deformity in *Melopsittacus undulatus* caused by *Knemidocoptes pilae*. Turkish Journal of Veterinary and Animal Sciences, 38(3), 344-346. https://doi.org/10.3906/vet-1311-36

2. Anderson, P. K. (2010). Human-Bird Interactions (Part I-Captive Birds). In: I. J. H. Duncan & P. Hawkins (Eds.). The Welfare of Domestic Fowl and Other Captive Birds, (vol. 9, pp. 17-51). Springer.

3. Arends, J. J. (2008). External parasites and poultry pests. In: Y. M. Saif (Ed.). Diseases of poultry, (12th ed., pp. 905-930). Blackwell Publishing.

4. Boseret, G., Losson, B., Mainil, J. G., Thiry, E. & Saegerman, C. (2013). Zoonoses in pet birds: Review and perspectives. Veterinary Research, 44(1), 36, 1-17. https://doi.org/10.1186/1297-9716-44-36

5. De Luna, C. J., Arkle, S., Harrington, D., George, D. R., Guy, J. H. & Sparagano, O. A. (2008). The poultry red mite *Dermanyssus gallinae* as a potential carrier of vector-borne diseases. Annals of the New York Academy of Sciences, 1149, 255-258. https://doi.org/10.1196/annals.1428.085

6. Dik, B. (2010). Türkiye'deki Bazı Kuş Türlerinden Yeni Çineyici Bit (Phthiraptera) Türü Kayıtları. Türkiye Parazitoloji Dergisi, 34, 168-173.

7. Dik, B., Kirpik, M. A., Sekercioglu, C. & Sasmaz, Y. (2011). Chewing Lice (Phthiraptera) Found on Songbirds (Passeriformes) in Turkey. Türkiye Parazitoloji Dergisi, 35(1), 34-39. https://doi.org/10.5152/tpd.2011.09

8. Dik, B., Albayrak, T., Adanir, R. & Uslu, U. (2013). Chewing Lice (Phthiraptera; Ischnocera, Amblycera) Species Found on Some Songbirds (Aves: Passeriformes). Kafkas Universitesi Veteriner Fakültesi Dergisi, 19(5), 755-760. DOI: 10.9775/kvfd.2013.8740

9. Ciloglu, A., Yildirim, A., Onder, Z., Yetismis, G., Duzlu, O., Simsek, E. & Inci, A. (2020). Molecular characterization of poultry red mite, *Dermanyssus gallinae* lineages in Turkey and first report of *Plasmodium* species in the mite populations. International Journal of Acarology, 46(4), 241-246. https://doi.org/10.1080/01647954.2020.1758775

10. Cristofani, P., Rossi, G., Fichi, G., Galosi, L. & Perrucci, S. (2017). New records of parasıtes in captive canaries (*Serinus canaria*) in Italy. 3rd ICARE - International Conference on Avian Herpetological and Exotic mammal medicine, March 25th - 29th, 2017, Venice, Italy.

11. Fard, M. V., Sharifi, S. F., Ganjali, M., Jahantigh, M. & Lopez-Aban, J. (2020). Identification of ectoparasites of ornamental birds in the north of Sistan and Baluchestan (southeast Iran). Iranian Journal of Veterinary Science and Technology, 12(2), 68-72. https://doi.org/10.22067/veterinary.v12i2.87675

12. Gothe, R. & Neitz, A. W. H. (1991). Tick paralysis: pathogeneses and etiology. Advances in Disease and Vector Research, 8, 177-204. In: Harris, K. F. (Ed.). Advances in Disease and Vector Research (pp. 177-204), Springer.

13. Hamstra, T. L. & Badyaev, A. V. (2009).Comprehensive investigation of ectoparasite community and abundance across life history stages of avian host. Journal of Zoology, 278(2), 91-99. https://doi.org/10.1111/j.1469-7998.2008.00547.x

14. Heylen, D. & Matthysen, E. (2008). Effect of tick parasitism on the health status of a passerine bird. Functional Ecology, 22, 1099-1107. https://doi.org/10.1111/j.1365-2435.2008.01463.x

15. Kehlmaier, C. & Quaisser, C. (2013). First Record of Ectoparasitic Insects on the Canarian Houbara Bustard (Gruiformes: Otididae). Annales Zoologici, 63, 511-515. https://doi.org/10.3161/000345413X672519

16. Koc, N., Yucesan, B. & Nalbantoglu, S. (2017). Papağanlarda *Dermanyssus gallinae* kaynaklı kanibalizm. Mehmet Akif Ersoy Üniversitesi Veteriner Fakültesi Dergisi, 2(2), 147-151. DOI: 10.24880/maeuvfd.363443

17. Kounek, F., Sychra, O., Capek, M., Lipkova, A. & Literak, I. (2011). Chewing lice of the genus Myrsidea (Phthiraptera: Menoponidae) from the Cardinalidae, Emberizidae, Fringillidae, and Thraupidae (Aves: Passeriformes) from Costa Rica, with descriptions of four new species. Zootaxa, 3032, 1-16. https://doi.org/10.11646/zootaxa.3032.1.1

18. Livinius, N. H., Debi-Dore, J. D., Ombugadu, A., Dibal, M. & Mafuyai, M. J. (2018). Survey of Ectoparasites Infesting Captive Birds in the Jos Museum Zoological Garden, North Central, Nigeria. Journal of Natural Sciences Research, 8(7), 36-40.

19. Marchiondo, A. A. & Endris, R. G. (2019). Arachnida. In: A. A. Marchiondo, L. R. Cruthers & J. J. Fourie (Eds.). Parasiticide Screening, (vol. 1, pp. 257-377), Academic Press.

20. Moller, A. P., Allander, K. & Dufva, R. (1990). Fitness Effects of Parasites on Passerine Birds: A Review. In: J. Blondel, A. Gosler, J. D. Lebreton, R. McCleery (Eds.). Population Biology of Passerine Birds (vol. 24, pp. 269-280). Springer.

21. Moller, A. P. & Lope, F. (1999). Senescence in a short-lived migratory bird: age-dependent morphology, migration, reproduction and parasitism. Journal of Animal Ecology, 68(1), 163-171. https://doi.org/10.1046/j.1365-2656.1999.00274.x

22. Moodi, B., Aliabadian, M., Moshaverinia, A. & Mirshamsi Kakhki, O. (2013). New data on the chewing lice (Phthiraptera) of passerine birds in East of Iran. Scientia Parasitologica, 14(2), 63-68.

23. Moss, W. W. (1968). An Illustrated Key To Species Of The Acarine Genus *Dermanyssus*. Journal of Medical Entomology, 5(1), 67-84. https://doi.org/10.1093/jmedent/5.1.67

24. Mullen, G. R. & Oconnor, B. M. (2002). Mites (Acari). In: L. A. Durden & G. R. Mullan (Eds.). Medical and Veterinary Entomology (pp. 449-516), Elsevier.

25. Musa, S., Afroz, S. D. & Khanum, H. (2012). Occurence of ecto- and endo parasites in pigeon (*Columba livia* Linn.). University Journal of Zoology, Rajshahi University, 30, 73-75. https://doi.org/10.3329/ujzru.v30i0.10758

26. Negm, M., Mohamed, A., El-Gepaly, H. & Abdelaziz, S. (2018). Mesostigmata mites (Acari: Parasitiformes) associated with birds and their nests from. Turkish Journal of Zoology, 42(6), 722-731. https://doi.org/10.3906/zoo-1801-24

27. Ombugadu, A., Echor, B. O., Jibril, A. B., Angbalaga, G.

A., Lapang, M. P., Micah, E. M., Njila, H. L., Isah, L., Nkup, C. D., Dogo, K. S. & Anzaku, A. A. (2019). Impact of Parasites in Captive Birds: A Review. Current Research in Environment and Biodiversity, 01, 1-12.

28. Prathipa, A., Jayathangaraj, M. G., Gomathinayagam, S. & Thangavelu, A. (2013). Feather Mites of Captive Psittacine Birds in Major Zoos of India. The Indian Veterinary Journal, 90(12), 92-93.

29. Prathipa, A., Jayathangaraj, M. G., Gomathinayagam, S. & Thangavelu, A. (2015). Feather Mites of Psittacine Birds. International Journal of Veterinary Science, 4(1), 30-32.

30. Proctor, H. & Owens, I. (2000). Mites and birds: diversity, parasitism, and coevolution. Trends in Ecology and Evolution, 15(9), 358-364. https://doi.org/10.1016/s0169-5347(00)01924-8

31. Saranya, K., Prathaban, S., Senthilkumar, K., Srithar, A. & Gomathinayagam, S. (2018). Prevalence of Ectoparasites and Its Identification in Captive Wild Birds. International Journal of Current Microbiology Applied Sciences, 7(8), 1093-1100. https://doi.org/10.20546/ijcmas.2018.708.124

32. Shah, A. H., Khan, M. N., Iqbal, Z. & Sajid, M. S. (2004). Review: Tick Infestation in Poultry. International Journal of Agriculture and Biology, 6(6), 1162-1165.

33. Sommer, D., Heffels-Redmann, U., Kohler, K., Lierz, M. & Kaleta, E. F. (2016). [Role of the poultry red mite (*Dermanyssus gallinae*) in the transmission of avian influenza A virus]. Tierarztliche Praxis. Ausgabe G, Grosstiere/Nutztiere, 44(1), 26-33. https://doi.org/10.15653/TPG-150413

34. Sparagano, O. A., George, D. R., Harrington, D. W. Giangaspero, A. (2014). Significance and control of the poultry red mite, *Dermanyssus gallinae*. Annual Review of Entomology, 59(1), 447-466. https://doi.org/10.1146/annurev-ento-011613-162101

35. Sychra, O. (2005). Morphological variation of *Neopsittaconirmus gracilis* (Phthiraptera, Ischnocera) from budgerigar, *Melopsittacus undulatus*. Biologia, 60(2), 137-142.

36. Sychra, O., Naz, S. & Rizvi, S. A. (2007). New reord of *Afrimenopon waar* (Eichler) (Phthiraptera: Menoponidae) from budgerigar *Melopsittacus undulatus* (Psittaciformes: Psittacidae) from Karachi, Pakistan. Parasitology Research, 101, 505-509. https://doi.org/10.1007/s00436-007-0505-z

37. Sychra, O., Literak, I., Podzemny, P. & Benedikt, V. (2008). Insect ectoparasites from wild passerine birds in the Czech Republic. Parasite, 15(4), 599-604. https://doi.org/10.1051/parasite/2008154599

38. Tags, S. Z., Agbede, R. I. S. & Mohammed, B. R. (2020). First incidence of ectoparasites in Abuja Zoological Parks, Abuja, Nigeria. Annals of Parasitology, 66(4), 533-537. https://doi.org/10.17420/ap6604.295

39. Valiente Moro, C., De Luna, C. J., Tod, A., Guy, J. H., Sparagano, O. A. & Zenner, L. (2009). The poultry red mite (*Dermanyssus gallinae*): a potential vector of pathogenic agents. Experimental and Applied Acarology, 48, 93-104. https://doi.org/10.1007/s10493-009-9248-0

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Research Article

A fast protein liquid chromatography method for purification of myoglobin from different species

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ABSTRACT

The aim of this study was to describe a fast method for the purification of high-purity myoglobin for mass spectroscopy analyses and to use it as standard grade material. A three-step fast protein liquid chromatography (FPLC) method was used to produce high-purity myoglobin. SEC 650 gel filtration followed by ENrich Q anion exchange chromatography was used to produce myoglobin in acceptable purity for most research methods. A second filtration step was carried out with narrow field SEC 70 gel to prepare high-purity myoglobin at standard-grade purity and suitable for mass spectroscopy analyses. At least 90% pure myoglobin was obtained by applying two chromatography steps to the samples of three species, and over 99% pure myoglobin was obtained in standard material quality and suitable for mass spectroscopy when the additional narrow field SEC 70 chromatography step was applied. The proposed method provided higher purity compared to other methods and takes less time. FPLC columns significantly reduced the duration of the chromatography steps. At the same time, the use of solid extraction columns instead of dialysis reduced the long overnight dialysis process to a few minutes.

INTRODUCTION

Myoglobin, a cytoplasmic protein found in cardiac and skeletal muscle, is a one-polypeptide chain of 154 amino acids with a molecular weight between 14 and 18 kDa (Ordway and Garry, 2004). Myoglobin's primary structure varies among species. These differences in amino acid sequences affect the secondary structure as well as the chemical behavior of proteins like polarity and isoelectric point. The characterization of myoglobin from various animals has become an important subject in nutrition, food science, and biology. The amino acid sequence of myoglobin from various species and edible animals was identified previously. The primary structure of myoglobin from livestock and edible animals like cattle, sheep, pigs, horses, rabbits, goats, bison, buffalo, musk oxen, chickens, turkeys, pheasants, tuna, mackerel, and sardine were established and generally used for food safety and meat adulteration and authentication(Di Giuseppe et al., 2018; Dosi et al., 2006; Joseph et al., 2011; Joseph, et al., 2010a; Mottola et al., 2022; Nurilmala et al., 2018; Ragucci et al., 2019; Saud et al., 2019; Suman et al., 2009; Tan et al., 2021)containing the heme pigment, is present in large amount in meat, thus influencing meat color and consumer choice. Here, the primary structure of Mb isolated from muscle of Phasianus colchicus L. was determined by using a comparative peptide mapping approach based on MALDI-TOF mass spectrometry. This strategy allowed the determination of common pheasant Mb primary sequence, which resulted identical to the chicken Mb, as also confirmed by intact molecular mass determination by ESI/Q-TOF mass spectrometry. Indeed, the accurate molecular mass (17,290.50 Da. Suman et al. compared the amino acid sequence of goat myoglobin with other ruminant myoglobin sequences (sheep, cattle, buffalo, and deer). It was similar to other ruminant myoglobin by having 153 residues. However, the amino acid sequence in goat myoglobin was quite different from that in sheep and other ruminants. The similarity between the myoglobin of the five tested animals was between 95.4% and 99.3% (Suman et al., 2009)(Table 1).

Table 1. Percentage sequence similarities among myoglobins from different meat-producing ruminant species. Goat (Capra hircus); Sheep (Ovis aries); Cattle (Bos Taurus); Buffalo (Bubalus bubalis); Deer (Cervus elaphus) (Suman et al., 2009).

Species	Goat	Sheep	Cattle	Buffalo	Deer
Goat	100				
Sheep	98.7	100			
Cattle	97.4	98.7	100		
Buffalo	95.4	96.7	98.0	100	
Deer	98.0	99.3	98.0	96.1	100

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In addition to health and food safety prospects in myoglobin studies, it is also used widely in biological science as a marker in evolution and taxonomy research (Di Giuseppe et al., 2017; Enoki et al., 2008; Ragucci et al., 2022; Romero-Herrera et al., 1976). Purification of myoglobin is the first and main step in research based on the characterization of myoglobin either for health, nutrition, and food sciences or for biological research. Cellulose and Sephadex-based columns are widely used for the purification of myoglobin in research laboratories. Yamazaki et al. described a two-column method at alkaline pH for purification of oxymyoglobin with DEAE-cellulose and another column that was not indicated (Yamazaki et al., 1964). Similar time-consuming two-column methods were developed in the following years (Gotoh and Shikama, 1974; Shikama and Sugawara, 1978; Suzuki et al., 1980). Later Renerre et al. described a two-step chromatographic method with DEAE-Sepharose and TSK gel filtration columns (Renerre et al., 1992) and Gatellier used a one-step chromatographic method with an HPLC column for the purification of myoglobin (Gatellier et al., 1993). Two-step purification methods were still used for the purification of myoglobin with different columns and buffers (Chotichayapong et al., 2016; Dosi et al., 2012; Enoki et al., 2008; Giaretta et al., 2013). Although these methods are generally effective, they are time-consuming, with approximately two or three days needed to purify myoglobin from one species.

Herein we describe an FPLC method that took less than one day to produce high-purity myoglobin from different animals for mass spectroscopy (MS) analyses and standard preparation. In addition, we present a two-dimensional SDS-PAGE method for characterizing and distinguishing myoglobin of different animals from each other.

MATERIAL and METHODS

Materials

Cattle and chicken hearts were obtained from the local market. A pig's heart was obtained from a pork market in Istanbul. ENrich SEC 650 and ENrich SEC 70 gel filtration chromatography and ENrich Q anion exchange chromatography preparative FPLC columns, IEF strips, precast gradient electrophoresis gels, and chemicals for IEF and electrophoresis were obtained from Bio-Rad, USA. Amicon ultra-15 centrifugal filters were obtained from Merck Millipore, USA. PD-10 desalting columns were obtained from GE Healthcare, USA. Pure horse myoglobin, size exclusion chromatography standards, and other chemicals were obtained from Sigma-Aldrich, USA.

Methods

Heart homogenization and myoglobin extraction

The animal hearts were cut into small pieces and suspended in 50 mM potassium phosphate buffer containing 0.5 mM EDTA (pH 7.4) at a ratio of 10 ml of buffer to 1 ml of meat and homogenized using a blender/homogenizer on ice. The resulting homogenate was then centrifuged at 20,000 × *g* for 40 minutes. The supernatant was filtered with Miracloth to remove fats and then centrifuged at 15,800 × *g* for 15 minutes and the pellet was discarded. Supernatant fractions containing myoglobin were pooled for the further steps.

SEC 650 size exclusion chromatography

All chromatography assays were carried out at 4 °C. An ENrich SEC 650 (10 × 300 mm) gel filtration column was used for the first step of purification. The column was equilibrated with 10 mM Tris-HCl buffer (pH 8.6) at a flow rate of 0.4 ml/min prior to the experiment and all SEC 650 experiments were carried out under the same conditions.

Preparation of a standard chromatogram for the ENrich SEC 650 size exclusion column

Prior to the application of homogenate supernatant to the column, the standard plot for the ENrich SEC 650 column was prepared. A mixture of six pure protein fractions, namely amylase (200 kDa), alcohol dehydrogenase, (150,000 kDa), cattle serum albumin (66 kDa), carbonic anhydrase (29 kDa), horse

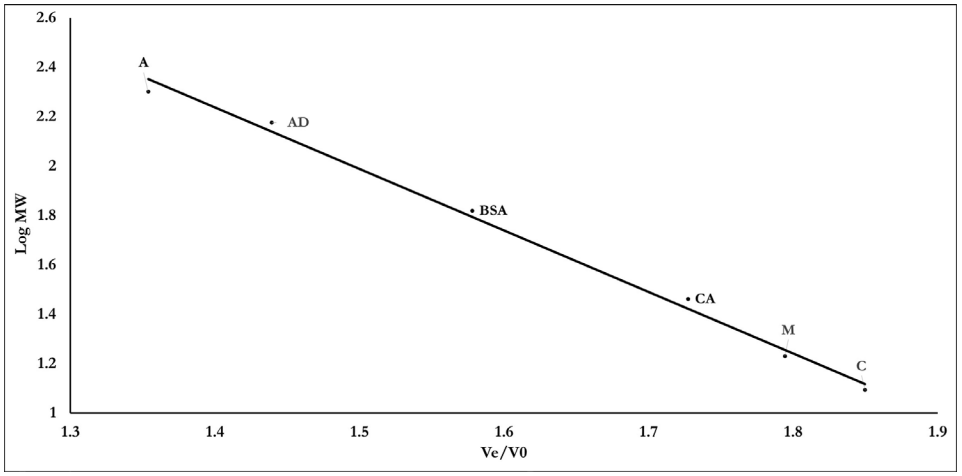

Figure 1. Standard plot for SEC 650 Gel Filtration chromatography column. Amylase 200 KDa(A), Alcohol Dehydrogenase, 150,000 KDa (AD), Cattle Serum Albumin 66KDa (BSA), Carbonic Anhydrase 29KDa (CA), Horse myoglobin (M) 17 KDa, Cytochrome C 12,4 KDa.(C)

myoglobin (17 kDa), and horse cytochrome C (12.4 kDa), was applied to the column. The void volume (V0) of the column and retention volumes (Ve) of the standards were determined and a standard plot was drawn as a logarithm of molecular weight (LogMW) versus V0/Ve. The retention volume of the myoglobin fraction determined was 15.55 ml (Figure 1).

SEC 650 size exclusion chromatography of meat samples

Supernatant from each animal heart was concentrated using Amicon Ultra-15 centrifugal filters with a cutoff membrane of 10 kDa to discard the light proteins below 10 kDa and reduce the volume of the sample to 1 ml, which is essential for gel filtration applications. Next 1 ml of the concentrated materials was applied to the column and the output of the column was monitored at 280 nm for total protein and 527 nm for myoglobin detection. The output was collected in 1-ml fractions and fractions near the retention time of 16 min with high absorbance at 527 nm were collected for the subsequent steps (Figure 2).

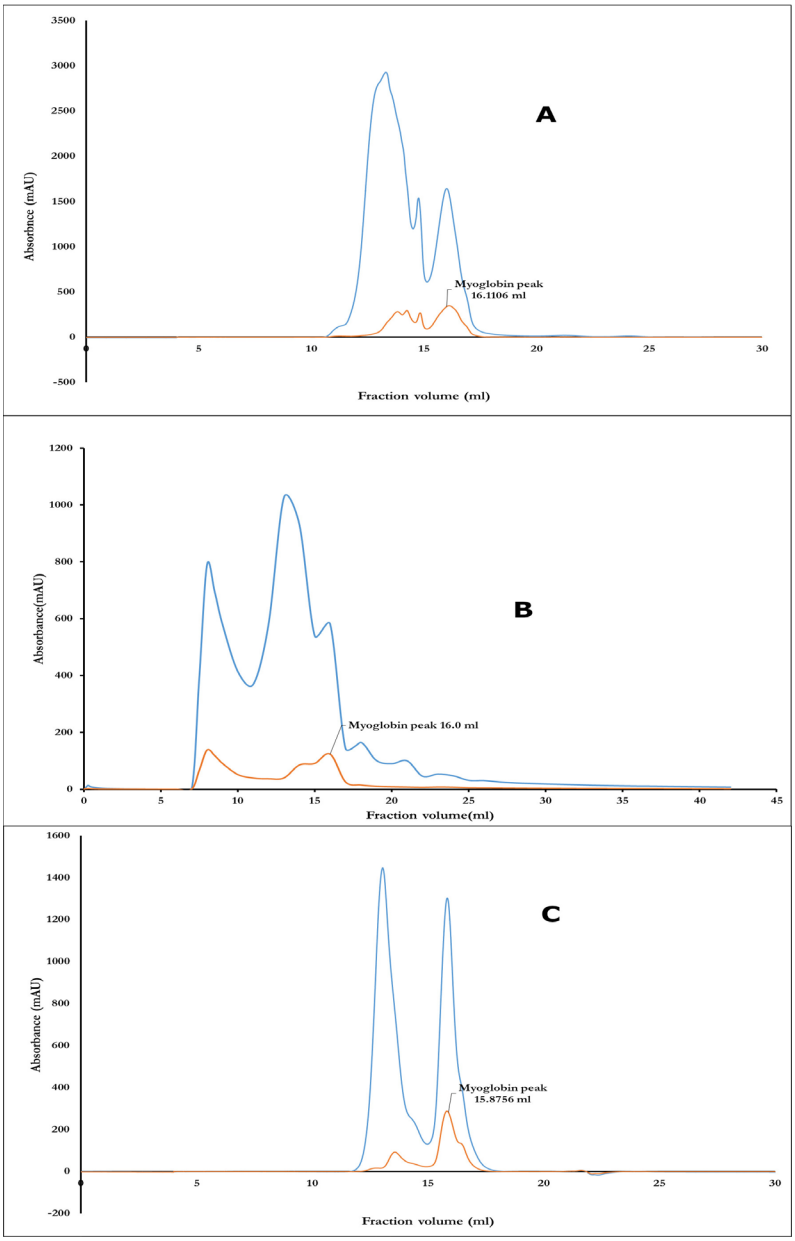

Figure 2. Size exclusion chromatogram of (A) cattle, (B) pork and (C) chicken myoglobin by SEC 650 FPLC column. (-Blue line: A280 , Brown line: A527)

ENrich Q anion exchange chromatography

Fractions collected from the size exclusion chromatography were then subjected to a PD-10 desalting column and the bonded material was eluted with 10 mM Tris-HCl buffer (pH 8.6) and applied to an ENrich Q anion exchange column (10 × 100 mm) equilibrated with the same buffer at a flow rate of 1 ml/min. The column was washed with the same buffer until A280 reached the baseline. Myoglobin was eluted from the column with a linear gradient of NaCl (0-1.0 M) in Tris-HCl (pH 8.6) buffer. The absorbance of the elusions at 280 nm and 527 nm was checked and fractions with high 527 nm absorbance were collected. Pure horse myoglobin was also subjected to

the same procedure for the determination of elution characteristics. Pig, cattle, horse, and chicken myoglobin were eluted at 0.20 M, 0.212 M, 0.256 M, and 0.370 M NaCl concentration of elution gradient, respectively. The myoglobin fractions were collected and stored for further steps (Figure 3).

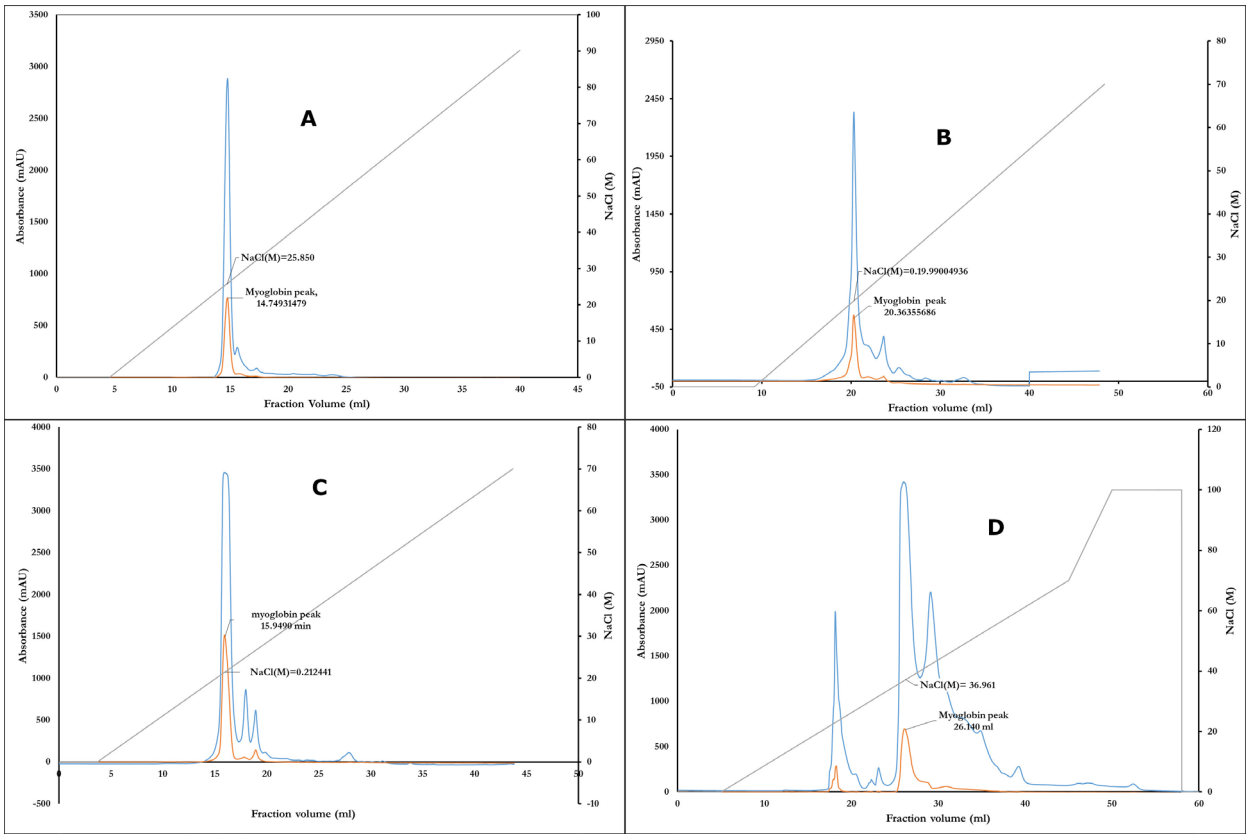

Figure 3. Anion Exchange chromatogram of (A) horse, (B) pork, (C) cattle and (D) chicken myoglobin by Enrich Q FPLC column. (¬Blue line: A280, Brown line: A527)

SEC 70 size exclusion chromatography of meat samples

The fractions collected from the IEX steps were applied to an ENrich SEC 70 column to remove the trace impurities and to prepare samples for the analytical steps. The column was equilibrated with 10 mM Tris-HCl buffer (pH 8.6) at a flow rate of 0.4 ml/min prior to the experiment and all SEC 70 experiments were carried out under the same conditions. Horse myoglobin was used as standard and all myoglobin fractions were eluted with 11.97-12.00 ml of elution buffer (Figure 4).

SDS-PAGE purity tests

The purity of samples was tested using SDS-PAGE. Samples were run on Bio-Rad "TGX any kD" precast gradient gels using the Laemmli buffer system (Laemmli, 1970).

Protein assay

Protein amounts in each myoglobin fraction were determined by the BCA method before mixing and application to the UHPLC system.

Characterization of myoglobin from different species by HPLC

UHPLC analysis of myoglobin was performed using a Thermo Fisher Dionex 3000 UHPLC system with a diode array detector. Myoglobin was characterized by the method described by Giaretta et al. with some modifications(Giaretta et al., 2013). A Protein-Pak Hi-Res Q column [4.6 × 100 mm, 5 µm (Waters SpA)] was used to separate myoglobin from different species. To load samples 80% 20 mM glycine NaOH buffer pH 9.2 (mobile phase A) and 20% 20 mM glycine NaOH buffer pH 9.2 containing 0.2 M NaCl (buffer B) were used. The myoglobin was eluted by a linear gradient of buffer B from 20% to 30% B for 7 min, followed by 70% for 2 min, returning to 20% for the following analyses. The analyses were carried out at a flow rate of 0.4 ml/min and 40 °C.

Two dimension electrophoresis of mixed myoglobin

Isoelectric focusing of the myoglobin mixture

IEF was performed using a Bio-Rad Protean i12 IEF system. A mixture of the 4 types of myoglobin studied was used as the sample. Myoglobin samples were dried and resuspended in an IEF rehydration solution containing 8 M urea, 2% CHAPS, 50 mM DTT, 0.2% carrier ampholyte, and 0.001% bromophenol blue to a final concentration of 1 mg protein in 1 ml solution. Next 200 µl of the sample was pipetted into a rehydration/equilibration tray and an IPG strip (pI +5-8) was placed over it side down. The strip was incubated for 12 hours for absorbing the sample. Ready strips were transferred to the IEF system focusing tray for the first dimension of electrophoresis. The IEF program was started with an application of 250 volts for 20 minutes and a gradual increase in voltage to

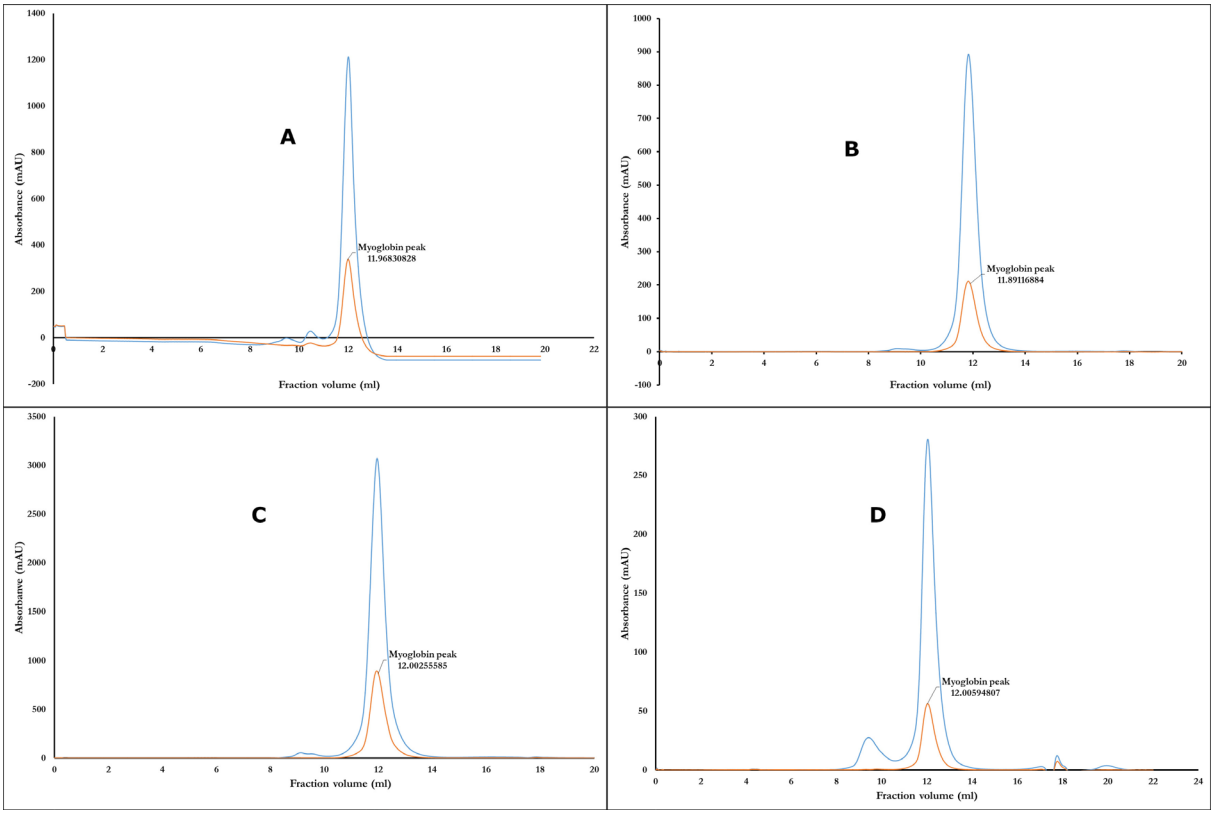

Figure 4. Size exclusion chromatogram of (A) horse, (B) cattle, (C) pork and (D) chicken myoglobin by SEC 70 FPLC column. (¬Blue line: A280, Brown line: A527)

8000 volts over 60 minutes. Power application continued until the total amount of voltage applied reached 26000 volt hours.

Second dimension SDS-PAGE

The IPG strips were equilibrated before the SDS-PAGE application. For this the proposed IPG strips were incubated in equilibration buffers I and II containing 6 M urea, 2% SDS, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, and 2% DTT for buffer I and 2.5% iodoacetamide for buffer II for 10 minutes each. The IPG strips then were placed on top of the SDS-PAGE gels. Continuity was achieved with overlay agarose containing 0.5% low melting point agarose in 25 mM tris, 192 mM glycine, 0.1% SDS, and a trace of bromophenol blue. Electrophoresis was carried out until the bromophenol blue reached the end of the SDS-PAGE gel. The Laemmli method was used for the second dimension electrophoresis (Laemmli, 1970). Gels were removed from their cassettes and stained with Coomassie brilliant blue R-250 stain.

RESULTS

Purification o myoglobin

Myoglobin from 3 species was purified partially by one step of gel filtration and one step of ion-exchange chromatography and purified completely by an additional step of narrow range gel filtration chromatography (Table 2). The purity of myoglobin obtained from the last step was also tested by SDS-PAGE (Figures 5 and 6).

Table 2. Purification steps of different animal myoglobin

	Cattle			Pork			Chicken		
Purification step	**purity (%)**	**(fold)**	**yield (%)**	**purity (%)**	**(fold)**	**yield (%)**	**purity (%)**	**(fold)**	**yield (%)**
Homogenate	25.69	1	100	22.85	1	100	24.72	1	100
Primary gel filtratio	77.84	3.03	75.43	80.2	3.51	83.65	80	2.89	80.7
Anyon Exchange	90.18	3.51	58.68	98.96	4.33	72.03	92.26	3.73	45.31
Secondary gel filtratio	99.81	3.89	48.44	100	4.38	71.27	99.1	4	30.7

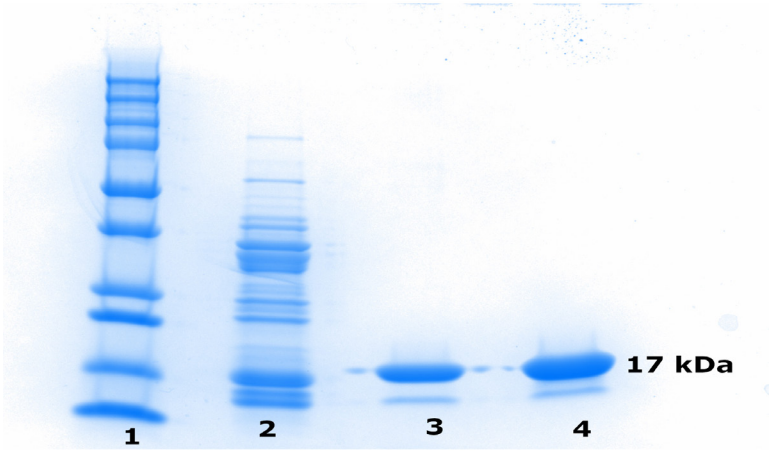

Figure 5. SDS-PAGE of myoglobins after anion exchange chromatography. 1: molecular wight Marker. 2: Chicken. 3: Cattle and 4: pork myoglobins

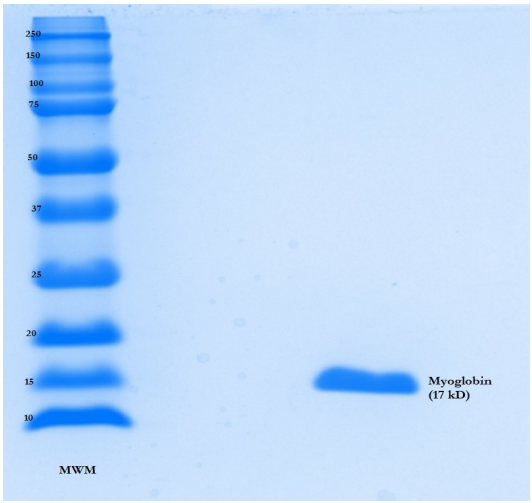

Figure 6. SSDS-PAGE of chicken myoglobin SEC 70 size exclusion chromatography. Left: molecular wight Marker. Right: Chicken myoglobin.

Characterizing of myoglobin samples

Myoglobin from 4 different species was thoroughly separated with HPLC. These results indicate that HPLC analyses of myoglobin could be used as an effective method for determining the species (Figure 7). Myoglobin fractions in the mixed

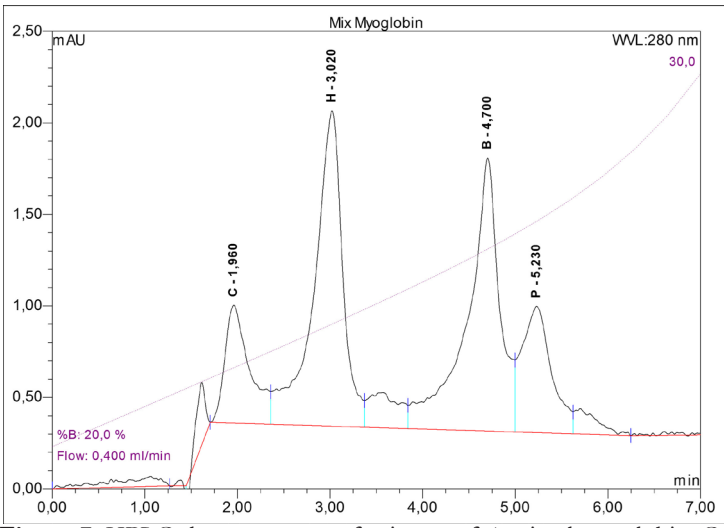

Figure 7. HPLC chromatogram of mixture of 4 animal myoglobin. C: Chicken, H: Equine, B: Bovine and p: porcine myoglobin peaks.

samples were also thoroughly separated and characterized by 2-dimensional gel electrophoresis due to differences in their iso-electric points (Figure 8) which also could be extracted from 2-dimensional gel electrophoresis (Table 3)

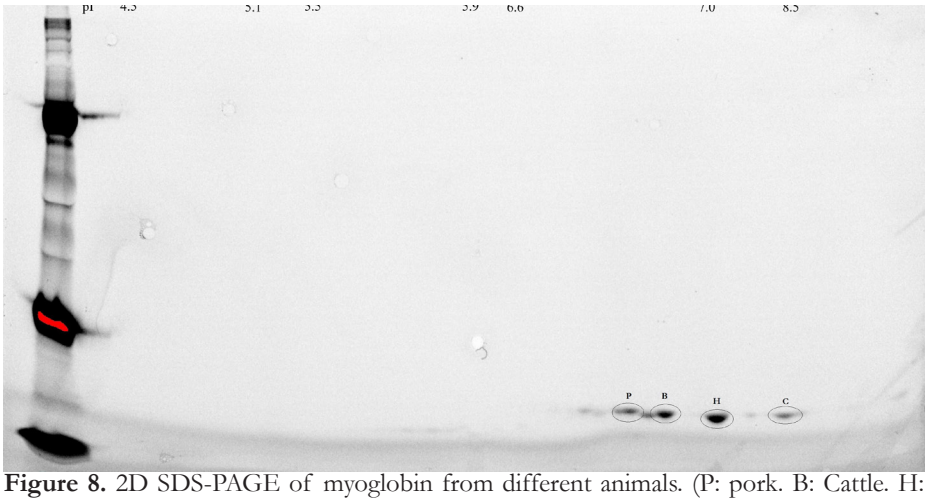

Figure 8. 2D SDS-PAGE of myoglobin from different animals. (P: pork. B: Cattle. H: Horse. C: chicken).

Table 3. Iso-electric points of myoglobin from pork, cattle, horse and chicken

Myoglobin	Isoelectric point
Pork	6.85
Cattle	6.89
Horse	7.12
chicken	7.95

DISCUSSION

Considering the results of the different purification steps, the purity of myoglobin for analysis in HPLC or 2D electrophoresis is acceptable after one step of gel filtration and one step of ion-exchange chromatography. These myoglobin fractions had trace impurities, so this level of purity is not sufficient for sequence analyses because of probable interference by extraneous peptides. The second gel filtration step with a narrow range gel filtration column was required for high-purity myoglobin production suitable for MS or sequence analyses. The results of both the HPLC and 2D SDS-PAGE analyses indicate that the products of this method meet the purpose of the study and could be used as standard materials for the determination of species in meat analyses in food safety and meat adulteration or any biological analyses.

CONCLUSION

The preparation of pure proteins is a major topic in biochemistry and many studies have been carried out to find an easy, feasible, and effective method for the purification of various proteins. In the present study, we introduced a fast and effective method for the purification of myoglobin as standard material for use in further analyses in species determination. The existing methods based on ammonium sulfate fractionation, dialysis and conventional low-pressure gel filtration, and ion exchange chromatography steps take days to prepare pure myoglobin(Chaijan et al., 2007; Joseph et al., 2010b), whereas in our method the purification time is reduced to a few hours. The repeatability and applicability of the procedure to different animal species are other advantages of our method. Unequal retention times in HPLC and separate points in 2D gel electrophoresis make myoglobin a favorable material in species determination. After specifying the position of myoglobin from each animal species by using standard myoglobin, a whole protein fraction of meat could be used after a simple protein isolation step. Special 2D gel analysis and comparison software is required for this purpose. The lack of this type of software limited our study in the case of studying mixed meat samples.

DECLARATIONS

Ethics Approval

There was not any human participant in this study.

Conflict o Interest

The authors have no conflicts of interest to declare

Consent for Publication

There was not any human participant in this study so no Consent for Publication is needed

Author contribution

Idea, concept and design: BS, NSM, ÖE

Data collection and analysis: BS, NSM, ÖE

Drafting of the manuscript: BS, NSM, ÖE

Critical review: BS, NSM, ÖE

Data Availability

Data were available on request from Correspondence author

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REFERENCES

1. Chaijan, M., Benjakul, S., Visessanguan, W., & Faustman, C. (2007). Characterisation of myoglobin from sardine (Sardinella gibbosa) dark muscle. Food Chemistry, 100(1), 156–164. https://doi.org/10.1016/j.foodchem.2005.09.030

2. Chotichayapong, C., Sattayasai, N., Kanzawa, N., Tamiya, T., Tsuchiya, T., & Chanthai, S. (2016). Purification, peptide mapping and spectroscopic characterization of myoglobin from striped snake-head fish (ophicephalus striatus). Oriental Journal of Chemistry, 32(1), 181–194. https://doi.org/10.13005/OJC/320119

3. Di Giuseppe, A. M. A., Russo, R., Ragucci, S., Landi, N., Rega, C., Chambery, A., & Di Maro, A. (2018). Myoglobin from common pheasant (Phasianus colchicus L.): Purification and primary structure characterization. Journal of Food Biochemistry, 42(2), 1–9. https://doi.org/10.1111/jfbc.12477

4. Di Giuseppe, A. M. A., Russo, L., Russo, R., Ragucci, S., Caso, J. V., Isernia, C., Chambery, A., & Di Maro, A. (2017). Molecular characterization of myoglobin from Sciurus vulgaris meridionalis: Primary structure, kinetics and spectroscopic studies. Biochimica et Biophysica Acta - Proteins and Proteomics, 1865(5), 499–509. https://doi.org/10.1016/j.bbapap.2017.02.011

5. Dosi, R., Carusone, A., Chambery, A., Severino, V., Parente, A., & Di Maro, A. (2012). Rapid primary structure determination of myoglobins by a complementary approach based on mass spectrometry and Edman degradation. Food Chemistry, 133(4), 1646–1652. https://doi.org/10.1016/j.foodchem.2012.02.042

6. Dosi, R., Di Maro, A., Chambery, A., Colonna, G., Costantini, S., Geraci, G., & Parente, A. (2006). Characterization and kinetics studies of water buffalo (Bubalus bubalis) myoglobin. Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology, 145(2), 230–238. https://doi.org/10.1016/j.cbpb.2006.07.006

7. Enoki, Y., Ohga, Y., Ishidate, H., & Morimoto, T. (2008). Primary structure of myoglobins from 31 species of birds. Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology, 149(1), 11–21. https://doi.org/10.1016/j.cbpb.2007.07.006

8. Gatellier, P., Anton, M., & Renerre, M. (1993). A rapid method of oxymyoglobin purification. Meat Science, 33(3), 401–407. https://doi.org/10.1016/0309-1740(93)90011-6

9. Giaretta, N., Di Giuseppe, A. M. A., Lippert, M., Parente, A., & Di Maro, A. (2013). Myoglobin as marker in meat adulteration: a UPLC method for determining the presence of pork meat in raw beef burger. Food Chemistry, 141(3), 1814–1820. https://doi.org/10.1016/J.FOODCHEM.2013.04.124

10. Gotoh, T., & Shikama, K. (1974). Autoxidation of native oxymyoglobin from bovine heart muscle. Archives of Biochemistry and Biophysics, 163(2), 476–481. https://doi.org/10.1016/0003-9861(74)90504-9

11. Joseph, P., Suman, S. P., Li, S., Beach, C. M., & Claus, J. R. (2010). Mass spectrometric characterization and thermostability of turkey myoglobin. LWT - Food Science and Technology, 43(2), 273–278. https://doi.org/10.1016/J.LWT.2009.08.019

12. Joseph, P., Suman, S. P., Li, S., Beach, C. M., Steinke, L., & Fontaine, M. (2010). Characterization of bison (Bison bison) myoglobin. Meat Science, 84(1), 71–78. https://doi.org/10.1016/j.meatsci.2009.08.014

13. Joseph, P., Suman, S. P., Li, S., Claus, J. R., Fontaine, M., & Steinke, L. (2011). Primary structure of turkey myoglobin. Food Chemistry, 129(1), 175–178. https://doi.org/10.1016/j.foodchem.2011.04.024

14. Laemmli, U. K. (1970). Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature, 227(5259), 680–685. https://doi.org/10.1038/227680a0

15. Mottola, A., Piredda, R., Catanese, G., Lorusso, L., Ciccarese, G., & Di Pinto, A. (2022). Species authentication of canned mackerel: Challenges in molecular identification and potential drivers of mislabelling. Food Control, 137(January), 108880. https://doi.org/10.1016/j.foodcont.2022.108880

16. Nurilmala, M., Ushio, H., Watabe, S., & Ochiai, Y. (2018). A streamlined isolation method and the autoxidation profiles of tuna myoglobin. Journal of Food Science and Technology, 55(5), 1641–1647. https://doi.org/10.1007/s13197-018-3068-3

17. Ordway, G. A., & Garry, D. J. (2004). Myoglobin: An essential hemoprotein in striated muscle. Journal of Experimental Biology, 207(20), 3441–3446. https://doi.org/10.1242/jeb.01172

18. Ragucci, S., Russo, R., Landi, N., Valletta, M., Chambery, A., Esposito, S., Raundrup, K., & Di Maro, A. (2019). Muskox myoglobin: purification, characterization and kinetics studies compared with cattle and water buffalo myoglobins. Journal of the Science of Food and Agriculture, 99(14), 6278–6286. https://doi.org/10.1002/jsfa.9901

19. Ragucci, S., Woodrow, P., Clemente, A., Russo, R., Valletta, M., Landi, N., Russo, L., Chambery, A., & Di Maro, A. (2022). Myoglobin from Atlantic and Tinker mackerels: Purification, characterization and its possible use as a molecular marker. International Journal of Biological Macromolecules, 214(June), 459–469. https://doi.org/10.1016/j.ijbiomac.2022.06.128

20. Renerre, M., Anton, M., & Gatellier, P. (1992). Autoxidation of purified myoglobin from two bovine muscles. Meat Science, 32(3), 331–342. https://doi.org/10.1016/0309-1740(92)90096-M

21. Romero-Herrera, A. E., Lehmann, H., Castillo, O., Joysey, K. A., & Friday, A. E. (1976). Myoglobin of the orangutan as a phylogenetic enigma. Nature 1976 261:5556, 261(5556), 162–164. https://doi.org/10.1038/261162a0

22. Saud, S., Li, G., Kong, H., Khan, M. I., Qiang, Z., Sun, Y., Liu, W., Ding, C., Xiao, H., Wang, Y., Li, H., & Cao, C. (2019). Identification of chicken meat quality via rapid array isoelectric focusing with extraction of hemoglobin and myoglobin in meat sample. Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences, 1128(August), 121790. https://doi.org/10.1016/j.jchromb.2019.121790

23. Shikama, K., & Sugawara, Y. (1978). Autoxidation of native oxymyoglobin. Kinetic analysis of the pH profile. European Journal of Biochemistry, 91(2), 407–413. https://doi.org/10.1111/J.1432-1033.1978.TB12693.X

24. Suman, S. P., Joseph, P., Li, S., Steinke, L., & Fontaine, M. (2009). Primary structure of goat myoglobin. Meat Science, 82(4), 456–460. https://doi.org/10.1016/j.meatsci.2009.02.017

25. Suzuki, T., Sugawara, Y., Satoh, Y., & Shikama, K. (1980). Human oxymyoglobin: isolation and characterization. Journal of Chromatography A, 195(2), 277–280. https://doi.org/10.1016/S0021-9673(00)96820-1

26. Tan, B., Sun, B., Sun, N., Li, C., Zhang, J., & Yang, W. (2021). Structure, functional properties and iron bioavailability of Pneumatophorus japonicus myoglobin and its glycosylation products. International Journal of Biological Macromolecules, 173, 524–531. https://doi.org/10.1016/j.ijbiomac.2021.01.138

27. Yamazaki, I., Yokota, K. N., & Shikama, K. (1964). Preparation of Crystalline Oxymyoglobin from Horse Heart. Journal of Biological Chemistry, 239(12), 4151–4153. https://doi.org/10.1016/S0021-9258(18)91147-6

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Research Article

Oleuropein attenuates placental growth factor expression by regulating oxidative stress and apoptosis in acrylamide hepatotoxicity

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ABSTRACT

The liver is susceptible to toxic effects, as it is the main site of acrylamide biotransformation and detoxification. Researchers have claimed that placental growth factor (PlGF) and its pathway are potentially involved in numerous diseases, including liver fibrosis and angiogenesis. Oleuropein is a natural phenolic compound with potent antioxidant effects. The purpose of this study was to examine the role of PlGF and the potential protection provided by oleuropein in acrylamide hepatotoxicity. Wistar albino rats were assigned into control, acrylamide (ACR) (5 mg/kg), oleuropein (OLE) (4.2 mg/kg), and ACR+OLE groups. Acrylamide and oleuropein were administered for 21 days. The control group received only physiological saline. Liver tissues were evaluated histologically and immunohistochemically. Histological examinations revealed significant enlargement of the sinusoidal vessels and abundant hepatocytes with pyknotic nuclei in the ACR group. Acrylamide toxicity resulted in elevated PlGF, accumulation of 8-hydroxydeoxyguanosine (8-OHdG), and increased Caspase-3 immunoreactivity in the liver. Oleuropein treatment reduced the increased expression of PlGF, 8-OHdG, and Caspase-3 against these deleterious effects observed in the ACR group. A positive correlation was observed between PlGF levels as well as oxidative stress and apoptosis markers in acrylamide toxicity. Oleuropein probably counteracted this mechanism by exhibiting antioxidant activity.

INTRODUCTION

Acrylamide was first discovered in 1893, and in 2002 researchers reported its presence in commonly consumed foodstuffs (Omar et al., 2015). Acrylamide is classified as a "possible carcinogen" and is found in heat-treated products, as well as cigarettes, soaps, plastic products, cleaning products, and baby foods (Esposito et al., 2021; Pietropaoli et al., 2022). Exposure to acrylamide causes toxic effects in the digestive tract, liver, kidney, heart, lung, and brain. A previous experimental study reported elevation in total oxidative state and malondialdehyde (MDA) levels in the rat liver following 21-day exposure to acrylamide, while the expression level of some antioxidant enzymes decreased (Rifai and Saleh, 2020). Acrylamide can also cause dysfunction and considerable reactive oxygen species (ROS) production by altering the mitochondrial membrane potential of hepatocytes (Seydi, 2015). Exposure to acrylamide in animal models results in several ultrastructural abnormalities with increases in MDA and reduced glutathione (GSH) and superoxide dismutase (SOD) levels in liver tissue (Gao et al., 2022). This means that the liver is directly affected by oxidative stress.

Although numerous studies have addressed acrylamide hepatotoxicity from different perspectives, the manner in which placental growth factor (PlGF) expression changes has not been previously examined. Acting as a pleiotropic cytokine, PIGF stimulates the growth, migration, and survival of endothelial cells and promotes pathological angiogenesis and wound healing (Li et al., 2017). Nutritional factors may cause changes in the liver, such as hepatocyte degeneration, necrosis, and replacement of the parenchyma by fibrotic tissue, resulting in loss of function. These events actually occur as a result of the activation of proinflammatory and profibrotic pathways. On the other hand, PlGF pathway blockade was found to cause no harm to healthy blood vessels, and pathological angiogenesis decreased (Li et al., 2017). Increased expression of PlGF has been shown in cirrhotic liver and hepatocellular carcinoma in both human and rodent models (Dewerchin and Carmeliet, 2012). Inhibition of PIGF has been shown to suppress liver fibrogenesis, reduce portal hypertension, and inhibit hepatocellular carcinoma (Heindryckx et al., 2013).

Polyphenols are natural antioxidants and chemoprotective agents that exhibit protective effects against diseases associated with oxidative stress and mitochondrial dysfunction. Oleuropein is the most important bioactive phenolic glycoside in olive leaves (Topuz, 2022). It exhibits powerful antioxidant and anti-apoptotic effects in several diseases (Alarcon de la Lastra, et al., 2001). On the other hand, inhibition of oxidative stress development can prevent structural and functional abnormalities in the liver. Various studies have reported strong evidence that oleuropein exerts a hepatoprotective effect (Yoon, 2018).

This study investigated the relationship between PlGF expression and oxidative stress, and the possible protective action of oleuropein, a subject which has not been investigated in previous studies of acrylamide-induced liver toxicity.

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MATERIAL and METHODS

Experimental Design

Twenty-four Wistar albino rats were assigned into four groups - control, oleuropein-treated (OLE group - 4.2 mg/kg), acrylamide (ACR group - 5 mg/kg acrylamide BioShop, Cat No. 79.06.1), and oleuropein+acrylamide (ACR+OLE group - 5 mg/kg ACR+ 4.2 mg/kg OLE). The control group was given saline solution only. All animals received reagent for 21 days in saline solution via oral gavage. All animal procedures were approved and supervised by the Balıkesir University Ethical Committee (ethical approval number 2022/10-2 with dated 05.01.2023).

On the 21st day of the experiment, the animals were anesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg) and sacrificed by cervical dislocation.

Oleuropein was obtained from Southern Marmara olive tree leaf extract. The amount of oleuropein in 100 mg/ml extract was determined as 4.2 mg using the HPLC method (Sharif et al., 2021)

Histological procedure

Ten percent neutral buffered formalin solution was used to fix the liver tissues. For histological tissue processing, tissues were washed overnight in running water and embedded in paraffin after dehydration and xylene clearing. Five-micrometer sections were taken from the tissues in the paraffin blocks by means of a microtome (Leica, Multicut, Germany) and stained with hematoxylin and eosin. Finally, histopathological changes in the liver were evaluated and photographed under light microscopy (Zeiss Axiolab 5, Jena, Germany).

Immunohistochemical procedure

Five micrometer-thick sections were taken from the paraffin blocks. After deparaffinization, these were incubated with citrate buffer for antigen retrieval. They were then treated with 3 % hydrogen peroxide for inhibition of endogenous peroxidase activity. Ten percent normal goat serum was used for non-specific binding inhibition.

The primary antibodies PlGF, 8-OHdG, and Caspase-3 (PlGF 1:250, NBP2-67067, Novus Biologicals; 8-OHdG: 1:400, BS-1278R, Bioss; Caspase-3: 1:250, BS-0081R, Bioss) were next incubated with the liver sections for one night and subsequently treated for 60 min at room temperature with the horseradish peroxidase-conjugated secondary antibody. Immunostaining was visualized with fresh DAB solution. The sections were finally counterstained with Gill's hematoxylin and mounted. The staining results were evaluated under a light microscope (Zeiss Axiolab 5, Jena, Germany).

Assessment of immunohistochemical reactivity

The histoscore (H-score) of the semi-quantitative staining intensities was obtained by evaluating the proportion of positive cells and the degree of staining (0, unstained; 1, weak; 2, median; and 3, strong), and values between 0 and 300 were found. The expression level of each antibody was subjected to statistical evaluation based on the median H-score value. Evaluations were performed by two different researchers (Numata et al., 2013).

Statistical Analysis

The study data were expressed as mean ± standard deviation and analyzed on SPSS software package (version 22; SPSS Inc., Chicago, IL, USA). Significance differences were determined by means of One-Way ANOVA and Tukey's multiple comparison post-hoc test. *$p<0.05$ and **$p<0.01$, as appropriate, were regarded as statistically significant.

RESULTS

Acrylamide causes serious histopathological changes in the liver

The histopathological findings for the study groups' H&E-stained liver tissues sections are given in Figure 1. Accordingly, while the tissues from the control group exhibited a normal histological appearance, a few cells in the OLE group exhibited acidophilic staining (thin arrows). In the ACR group, the presence of diffuse acidophilic staining cells around the portal vein was noteworthy, and these cells were thought to be necrotic. Additionally, dilated sinusoids surrounding the dilated central vein were noted (arrow heads). The cytoplasmic borders of hepatocytes were unclear, and many contained pyknotic nuclei (thick arrow).

Oleuropein lowered increased PlGF immune expression in liver tissues exposed to acrylamide

The H-scores of the angiogenic marker PlGF were examined immunohistochemically in liver sections from all the experimental group, as shown in Figure 2A. Statistical analysis of the positive PlGF- stained cells in liver sections revealed that exposure to acrylamide (291 ± 25.88) significantly increased PlGF expression in the liver compared to the control group (118 ± 18.17) ($p < 0.01$). However, significant decreases were found in the ACR+OLE (145 ± 16.75) and OLE (123 ± 13.54) groups compared to the ACR group ($p < 0.01$) (Figure 2A and Figure 3).

Oleuropein attenuates acrylamide-induced liver apoptosis in rats

This study next investigated the roles of oleuropein in acrylamide-induced apoptosis. The results showed that oleuropein significantly inhibited the activation of apoptotic markers against the promotion of Caspase-3 in acrylamide application. Statistical analysis of the Caspase-3 positive stained cells in liver tissue are shown in Figure 2B.

The OLE group exhibited mild expression (140 ± 25.64). Acrylamide treatment caused a marked elevation in H-scores (296 ± 21) compared to the control group (91.6 ± 29.43), while a significant decrease was found in the ACR+OLE group H-scores (229 ± 17.17) compared to the ACR group ($p < 0.01$) (Figure 2B and Figure 4).

Oleuropein mitigates oxidative stress-induced DNA damage

Since DNA damage has been identified in case of acrylamide exposure, we stained liver tissue sections for 8-OHdG,

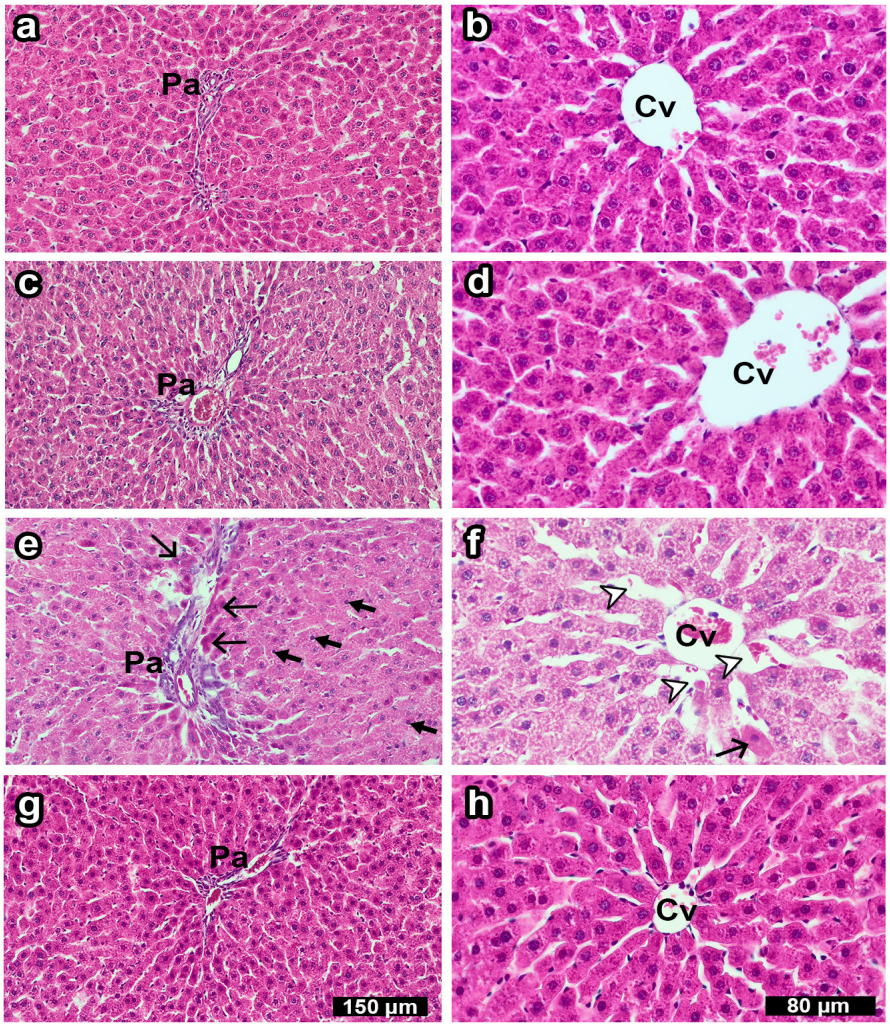

Figure 1. Photomicrographs representing H&E staining in liver sections. The control group (a, b) exhibits normal histological architecture in both the portal area (Pa) and central vein (Cv). Although acidophilic staining cells were noted in hepatocytes near the portal area in the OLE group (c), the structure of the central vein and surrounding hepatocytes were normal in appearance (d). However, in the ACR group, numerous hepatocytes with impaired integrity, unclear borders, and acidophilic staining (thin arrows) were observed near the portal area (e). Also, some hepatocyte nuclei have pyknotic nuclei (thick arrows) (e). The central vein was dilated, and dilatation was also present in the sinusoidal vessels between the hepatocyte cords (arrow heads) (e, f). However, oleuropein successfully protected the normal structure against the destructive effects of acrylamide in both the portal area and central vein structures in the ACR+OLE group (g, h). Paraffin, Scale bar: 150 µm and 80 µm for left and right panels.

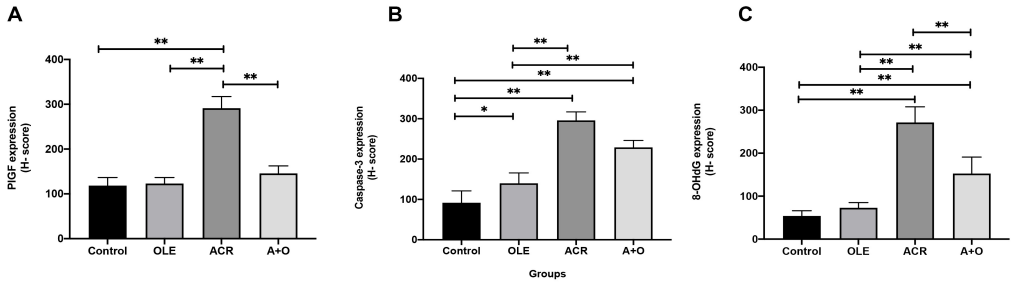

Figure 2. Statistical expression of the H-scores for PlGF, Caspase-3, and 8-OHdG expression in all the study groups. In the ACR group PlGF, Caspase-3, and 8-OHdG H-score was significantly increased compared to the other groups ($p < 0.01$). On the other hand, the A+O group H-score of PlGF and 8-OHdG was significantly decreased from the ACR group ($p < 0.01$). Besides this, the A+O group PlGF H-score did not differ from the control group ($p > 0.01$).

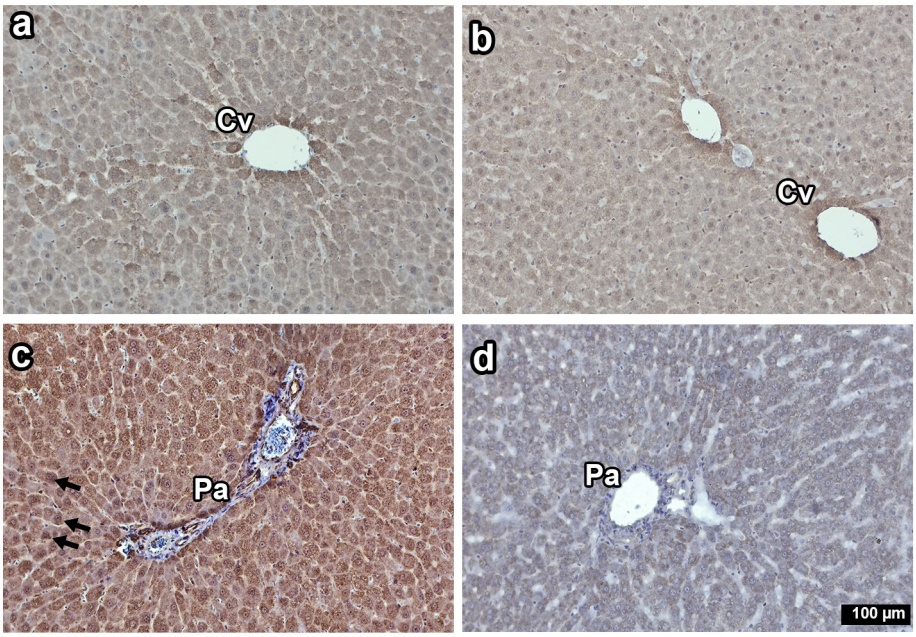

Figure 3. Immunohistochemical staining of PlGF in all four groups. Staining intensity of control, OLE and A+O groups (a, b, d) was similar. On the other hand, significantly increased cytoplasmic and nuclei staining of PlGF was observed in the ACR group (c). In addition, PlGF was widely expressed in most of the hepatic parenchyma of the ACR group and around the portal area. Arrow, positive stained cells; Pa, portal area; Cv, central vein. Strept-ABC, DAB, paraffin section. Scale bar: 100 µm for all panels.

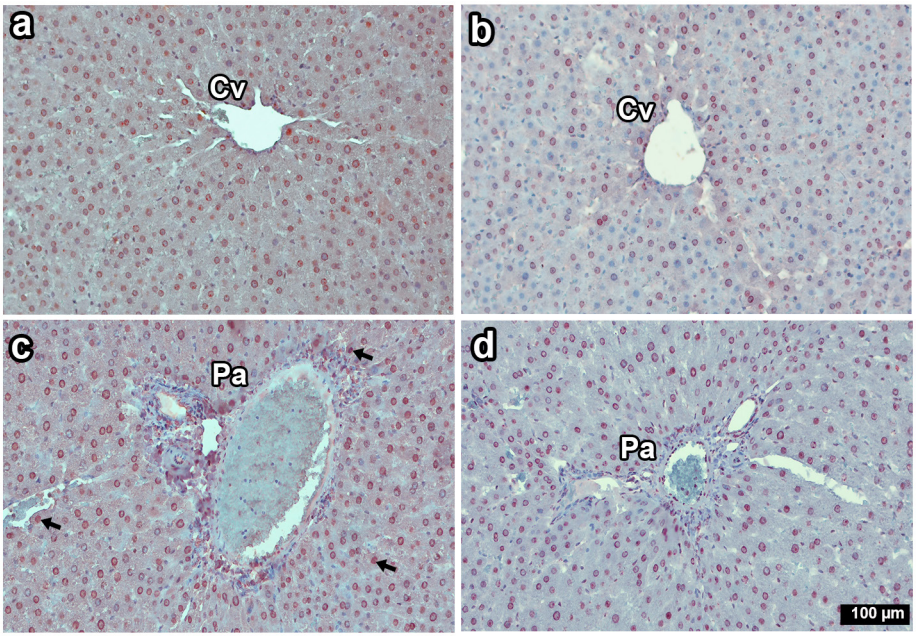

Figure 4. Immunohistochemical staining of Caspase-3 in all four groups. The control, OLE, and A+O groups (a, b, d) showed moderate immunoreactivity while increased cytoplasmic and nuclei staining of hepatocytes against the Caspase-3 antibody was observed in the ACR group (c). Arrow, positive stained cells; Pa, portal area; Cv, central vein. Strept-ABC, DAB, paraffin section. Scale bar: 100 µm for all panels.

a marker of oxidative stress-induced DNA lesions, in order to investigate the association between oleuropein and increased oxidative stress levels. Immunohistochemical analysis revealed stronger 8-OHdG staining in liver tissues (271 ± 36.53) in the ACR group compared to the control group ($p < 0.01$). In contrast, the OLE+ACR group liver tissue sections exhibited lower 8-OHdG expression (152 ± 38.35) than the ACR group ($p < 0.01$) (Figure 2C and Figure 5). It may therefore be concluded that oleuropein mitigates acrylamide-induced DNA damage.

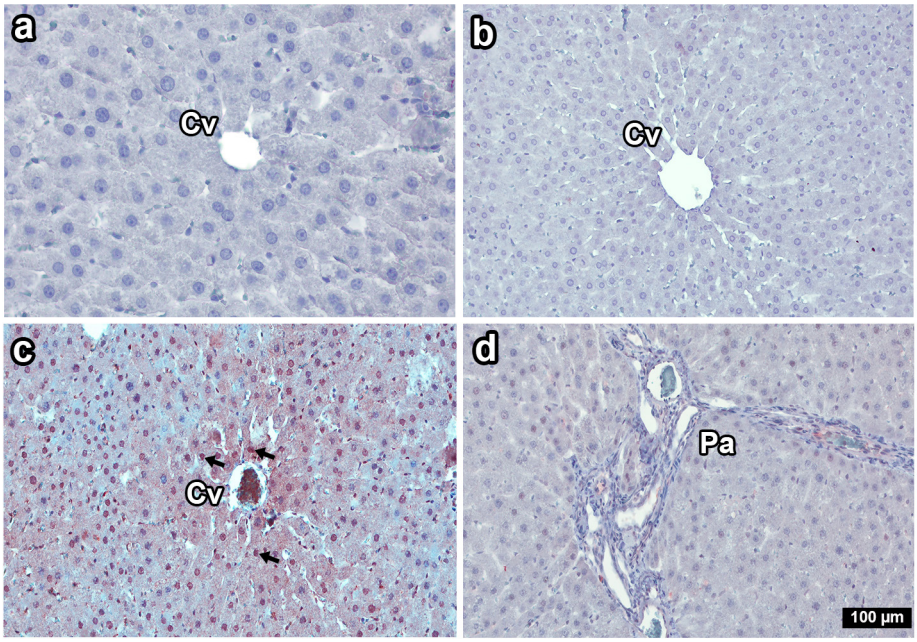

Figure 5. Immunohistochemical staining of 8-OHdG in all four groups. Staining intensity of control, OLE and A+O groups (a, b, d) was similar. On the other hand, it was determined that the expression of 8-OHdG increased significantly in the nucleus and cytoplasm of hepatocytes around the central vein. Arrow, positive stained cells; Pa, portal area; Cv, central vein. Strept-ABC, DAB, paraffin section. Scale bar: 100 µm for all panels.

DISCUSSION

The findings of this study revealed that oleuropein regulates PlGF levels by reducing Caspase-3 and 8-OHdG immunoreactivity, which increased notably with exposure to acrylamide. Our findings also support the idea that PlGF plays an important role in the pathogenesis of acrylamide hepatotoxicity and indicate that it may be a potential therapeutic target in acute acrylamide exposure. In addition, reduction of this angiogenic factor by oleuropein in acrylamide-exposed rats resulted in improvements in hepatic structures.

Increases PlGF in the liver is reported to cause liver damage, activation of monocytes and hepatic macrophages, fibrosis development, and hepatic inflammation (Li et al., 2017). An animal study in which PIGF was blocked by antibodies or genetic ablation reported decreased fibrogenesis and portal hypertension, as well as inhibition of the activation of hepatic stellate cells (Yano et al., 2008). In another study, Yano et al. reported that increased cytokine levels may contribute to an increase in PIGF levels (Yano et al., 2006). Various studies have reported that ACR supports inflammation, and that increased ROS levels promote a rise in the levels of proinflammatory cytokines (Ghorbel et al., 2015; Sengul et al., 2021). Another previous study reported significantly increased TNF-α and IL-1β levels in acrylamide-induced neurotoxicity (Santhanasabapathy et al., 2015). Researchers have also reported that, in addition to these cytokines, IL-6 also increased with acrylamide toxicity (Ghorbel et al., 2015). The increase in PlGF in the present study may have occurred through this mechanism. Although we did not measure proinflammatory cytokine levels, oxidative stress and DNA damage markers were correlated with increased PlGF in acrylamide exposure.

Oxidative stress plays a key role in the pathogenesis of acrylamide toxicity (Sengul et al., 2021; Uthra et al., 2022). The oxidative stress induced by acrylamide is related to changes in cellular antioxidant status. A previous study of acrylamide-induced nephrotoxicity observed that renal lipid peroxidation raised MDA levels and also resulted in a significant reduction of GSH, GPx, SOD, and CAT activities (Sengul et al., 2021). Significantly increased expression of Caspase-3, a key protease activated in the early period of apoptosis, is occurs in the sciatic nerves, spinal cord, and kidneys of rats treated with acrylamide (Li et al., 2006; Sengul et al., 2021). In the present investigation, and similarly to previous studies, acrylamide significantly increased the immunodensity of apoptosis protein Caspase-3 and stimulated apoptosis (Seydi, 2015). An increase was also determined in the immunoactivity of 8-OHdG, the principal indicator of DNA damage, in the ACR group. Similarly, there are studies showing marked 8-OHdG elevation in groups treated with acrylamide compared to normal healthy groups (Bin-Jumah et al., 2021; El-Beltagi, 2016).

The histopathological investigations in this study also corroborated the hepatotoxic effects of acrylamide. Previous studies have demonstrated that acrylamide causes severe histopathological changes in the liver (Gao et al., 2022; Gedik et al., 2017). Researchers reported that these changes in liver tissue are based on oxidative stress (Gedik et al., 2017). Our histopathological results also support this view corelated with literature.

In addition, in the present study, treatment with oleuropein after acrylamide intoxication reduced Caspase-3 and 8-OHdG expression levels in liver tissues. This may be attributed to the fact that oleuropein exhibits antioxidative and anti-inflammatory activities (Bakir et al., 2018). The hydroxyl groups (especially

the 1,2-dihydroxybenzene part) in the chemical structure of oleuropein can donate hydrogen to prevent oxidation, bestowing strong antioxidant activity (Hassen, 2015). In agreement with our findings, it has been proved in various experimental toxicology models that oleuropein is capable of reducing 8-OHdG formation and oxidative stress (Bakir et al., 2018; Koc et al., 2019). Numerous previous studies have also shown that oleuropein attenuates neuronal toxicity (Khalatbary and Ahmadvand, 2012; Khalatbary et al., 2015), myocardial damage and liver toxicity (Jemai et al., 2020) by mitigating several apoptotic factors (Manna et al., 2004).

Studies specifically addressing the hepatoprotective effects of oleuropein show that it attenuates 3-Nitrotyrosine formation, and NF-kB and Caspase-3 activation (Domitrovic et al., 2012). In addition, immunohistochemical analyses have confirmed that oleuropein regulates smooth muscle α-actin, toll-like receptor-4, NADPH oxidase, collagen α1 types I and III, transforming growth factor β1, and fibroblast growth factor receptor 1, finally reducing liver fibrosis and necrosis (Kim et al., 2014).

CONCLUSION

The findings of this study strongly support the idea that PlGF is a marker of acrylamide toxicity and correlates with increased apoptosis and DNA damage in liver tissue.

Taken together, our findings show that oleuropein reduces increased liver damage and PlGF expression levels induced by acrylamide treatment in rats. The mechanism underlying these effects may be at least to some degree related to the protection provided by oleuropein against acrylamide-induced apoptosis and DNA damage in the rat liver. On the other hand, it is a limitation in this study that the mRNA expression levels of PlGF, Caspase-3 and 8-OHdG have not been investigated. It is recommended to use western blot and qRT-PCR methods in studies to elucidate the underlying mechanisms.

DECLARATIONS

Ethics Approval

This study was approved by the Balıkesir University Ethical Committee (number 2022/10-2 with dated 05.01.2023).

Conflicts o Interest

The authors declare that they have no conflict of interest.

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Author Contribution

Idea, concept and design: MT

Data collection and analysis: MT, KKT

Drafting of the manuscript: KKT

Critical review: MT, KKT

Data Availability

The data used to prepare this manuscript are available from the corresponding author when requested.

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REFERENCES

1. Alarcon de la Lastra, C., Barranco M.D., Motilva V., & Herrerias J.M. (2001). Mediterranean diet and health: biological importance of olive oil. Current Pharmaceutical Design, 7, 933-950. https://doi.org/10.2174/1381612013397654

2. Bakir, M., Geyikoglu F., Koc K., & Cerig S. (2018). Therapeutic effects of oleuropein on cisplatin-induced pancreas injury in rats. Journal of Cancer Research and Therapeutics, 14, 671-678. https://doi.org/10.4103/jcrt.JCRT_1040_16

3. Bin-Jumah, M.N., Al-Huqail A.A., Abdelnaeim N., Kamel M., Fouda M.M.A., Abulmeaty M.M.A., Saadeldin I.M., & Abdel-Daim M.M. (2021). Potential protective effects of Spirulina platensis on liver, kidney, and brain acrylamide toxicity in rats. Environmental Science and Pollution Research, 28, 26653-26663. https://doi.org/10.1007/s11356-021-12422-x

4. Dewerchin, M., & Carmeliet P. (2012). PlGF: a multitasking cytokine with disease-restricted activity. Cold Spring Harbor Perspectives in Medicine, 2. https://doi.org/10.1101/cshperspect.a011056

5. Domitrovic, R., Jakovac H., Marchesi V.V., Sain I., Romic Z., & Rahelic D. (2012). Preventive and therapeutic effects of oleuropein against carbon tetrachloride-induced liver damage in mice. Pharmacology Research, 65, 451-464. https://doi.org/10.1016/j.phrs.2011.12.005

6. El-Beltagi, H.S., & Ahmed, M.M. (2016). Assessment the protective role of quercetin on acrylamide-induced oxidative stress in rats Journal of Food Biochemistry 40, 715–723. https://doi.org/10.1111/jfbc.12262

7. Esposito, F., Nolasco A., Caracciolo F., Velotto S., Montuori P., Romano R., Stasi T., & Cirillo T. (2021). Acrylamide in Baby Foods: A Probabilistic Exposure Assessment. Foods, 10. https://doi.org/10.3390/foods10122900

8. Gao, H., Xue Y., Wu L., Huo J., Pang Y., Chen J., & Gao Q. (2022). Protective Effect of Lycium ruthenicum Polyphenols on Oxidative Stress against Acrylamide Induced Liver Injury in Rats. Molecules, 27. https://doi.org/10.3390/molecules27134100

9. Gedik, S., Erdemli M.E., Gul M., Yigitcan B., Gozukara Bag H., Aksungur Z., & Altinoz E. (2017). Hepatoprotective effects of crocin on biochemical and histopathological alterations following acrylamide-induced liver injury in Wistar rats. Biomedicine Pharmacotherapy, 95, 764-770. https://doi.org/10.1016/j.biopha.2017.08.139

10. Ghorbel, I., Maktouf S., Kallel C., Ellouze Chaabouni S., Boudawara T., & Zeghal N. (2015). Disruption of erythrocyte antioxidant defense system, hematological parameters, induction of pro-inflammatory cytokines and DNA damage in liver of co-exposed rats to aluminium and acrylamide. Chemico-biological interactions, 236, 31-40. https://doi.org/10.1016/j.cbi.2015.04.020

11. Hassen, I., Casabianca, H., & Hosni, K. (2015). Biological activities of the natural antioxidant oleuropein: Exceeding the expectation–A mini-review. Journal of Functional Foods, 18, 926-940. https://doi.org/10.1016/j.jff.2014.09.001

12. Heindryckx, F., Coulon S., Terrie E., Casteleyn C., Stassen J.M., Geerts A., Libbrecht L., Allemeersch J., Carmeliet P., Colle I., & Van Vlierberghe H. (2013). The placental growth factor as a target against hepatocellular carcinoma in a diethylnitrosamine-induced mouse model. Journal of Hepatology, 58, 319-328. https://doi.org/10.1016/j.jhep.2012.09.032

13. Jemai, H., Mahmoudi A., Feryeni A., Fki I., Boullagui Z., Choura S., Chamkha M., & Sayadi S. (2020). Hepatoprotective Effect of Oleuropein-Rich Extract from Olive Leaves against Cadmium-Induced Toxicity in Mice. BioMed Research International, 2020, 4398924. https://doi.org/10.1155/2020/4398924

14. Khalatbary, A.R., & Ahmadvand H. (2012). Neuroprotective effect of oleuropein following spinal cord injury in rats. Neurological research, 34, 44-51. https://doi.org/10.1179/1743132811Y.0000000058

15. Khalatbary, A.R., Ghaffari E., & Mohammadnegad B. (2015). Protective Role of Oleuropein against Acute Deltamethrin-Induced Neurotoxicity in Rat Brain. Iranian Biomedical Journal, 19, 247-253. https://doi.org/10.7508/ibj.2015.04.009

16. Kim, S.W., Hur W., Li T.Z., Lee Y.K., Choi J.E., Hong S.W., Lyoo K.S., You C.R., Jung E.S., Jung C.K., Park T., Um S.J., & Yoon S.K. (2014). Oleuropein prevents the progression of steatohepatitis to hepatic fibrosis induced by a high-fat diet in mice. Experimental & Molecular Medicine, 46, e92. https://doi.org/10.1038/emm.2014.10

17. Koc, K., Cerig S., Ozek N.S., Aysin F., Yildirim S., & Cakmak O., Hosseinigouzdagani M. and Geyikoglu F. (2019). The efficacy of oleuropein against non-steroidal anti-inflammatory drug induced toxicity in rat kidney. Environmental Toxicology, 34, 67-72. https://doi.org/10.1002/tox.22658

18. Li, S.X., Cui N., Zhang C.L., Zhao X.L., Yu S.F., & Xie K.Q. (2006). Effect of subchronic exposure to acrylamide induced on the expression of bcl-2, bax and caspase-3 in the rat nervous system. Toxicology, 217, 46-53. https://doi.org/10.1016/j.tox.2005.08.018

19. Li, X., Jin Q., Yao Q., Zhou Y., Zou Y., Li Z., Zhang S., & Tu C. (2017). Placental Growth Factor Contributes to Liver Inflammation, Angiogenesis, Fibrosis in Mice by Promoting Hepatic Macrophage Recruitment and Activation. Frontiers in immunology, 8, 801. https://doi.org/10.3389/fimmu.2017.00801

20. Li, X., Yao Q.Y., Liu H.C., Jin Q.W., Xu B.L., Zhang S.C., & Tu C.T. (2017). Placental growth factor silencing ameliorates liver fibrosis and angiogenesis and inhibits activation of hepatic stellate cells in a murine model of chronic liver disease. Journal of Cellular and Molecular Medicine, 21, 2370-2385. https://doi.org/10.1111/jcmm.13158

21. Manna, C., Migliardi V., Golino P., Scognamiglio A., Galletti P., Chiariello M., & Zappia V. (2004). Oleuropein prevents oxidative myocardial injury induced by ischemia and reperfusion. The Journal of Nutritional Biochemistry, 15, 461-466. https://doi.org/10.1016/j.jnutbio.2003.12.010

22. Numata, M., Morinaga S., Watanabe T., Tamagawa H., Yamamoto N., Shiozawa M., Nakamura Y., Kameda Y., Okawa S., Rino Y., Akaike M., Masuda M., & Miyagi Y. (2013). The clinical significance of SWI/SNF complex in pancreatic cancer. International Journal of Oncology 42, 403-410. https://doi.org/10.3892/ijo.2012.1723

23. Omar, M.M., Elbashir A.A., & Schmitz O.J. (2015). Determination of acrylamide in Sudanese food by high performance liquid chromatography coupled with LTQ Orbitrap mass spectrometry. Food chemistry, 176, 342-349. https://doi.org/10.1016/j.foodchem.2014.12.091

24. Pietropaoli, F., Pantalone S., Cichelli A., & d'Alessandro N. (2022). Acrylamide in widely consumed foods - a review. Food additives and contaminants Part A, chemistry, analysis, control, exposure and risk assessment, 39, 853-887. https://doi.org/10.1080/19440049.2022.2046292

25. Rifai, L., & Saleh FA. (2020). A Review on Acrylamide in Food: Occurrence, Toxicity, and Mitigation Strategies. International Journal of Toxicology, 39, 93-102. https://doi.org/10.1177/1091581820902405

26. Santhanasabapathy, R., Vasudevan S., Anupriya K., Pabitha R., & Sudhandiran G. (2015). Farnesol quells oxidative stress, reactive gliosis and inflammation during acrylamide-induced neurotoxicity: Behavioral and biochemical evidence. Neuroscience, 308, 212-227. https://doi.org/10.1016/j.neuroscience.2015.08.067

27. Sengul, E., Gelen V., Yildirim S., Tekin S., & Dag Y. (2021). The Effects of Selenium in Acrylamide-Induced Nephrotoxicity in Rats: Roles of Oxidative Stress, Inflammation, Apoptosis, and DNA Damage. Biological trace element research, 199, 173-184. https://doi.org/10.1007/s12011-020-02111-0

28. Seydi, E., Rajabi, M., Salimi, A., & Pourahmad, J. (2015). Involvement of mitochondrial-mediated caspase-3 activation and lysosomal labilization in acrylamide-induced liver toxicity. Toxicological & Environmental Chemistry, 97, 563-575. https://doi.org/10.1080/02772248.2015.1047671

29. Sharif, K.O.M., Tufekci E.F., Ustaoglu B., Altunoglu Y.C., Zengin G., Llorent-Martínez E.J., Guney K. & Baloglu M.C. (2021). Anticancer and biological properties of leaf and flower extracts of Echinacea purpurea (L.) Moench. Food Bioscience, 41, 101005. https://doi.org/10.1016/j.fbio.2021.101005

30. Topuz, S., & Bayram, M. . (2022). Oleuropein extraction from leaves of three olive varieties (Olea europaea L.): Antioxidant and antimicrobial properties of purified oleuropein and oleuropein extracts. Journal of food processing and preservation, 46, e15697. https://doi.org/10.1111/jfpp.15697

31. Uthra, C., Reshi M.S., Jaswal A., Yadav D., Shrivastava

S., Sinha N., & Shukla S. (2022). Protective efficacy of rutin against acrylamide-induced oxidative stress, biochemical alterations and histopathological lesions in rats. Toxicology Research, 11, 215-225. https://doi.org/10.1093/toxres/tfab125

32. Valavanidis, A., Vlachogianni T., & Fiotakis C. (2009). 8-hydroxy-2' -deoxyguanosine (8-OHdG): A critical biomarker of oxidative stress and carcinogenesis. Journal of environmental science and health. Part C, Environmental carcinogenesis & ecotoxicology reviews, 27, 120-139. https://doi.org/10.1080/10590500902885684

33. Yano, K., Liaw P.C., Mullington J.M., Shih S.C., Okada H., Bodyak N., Kang P.M., Toltl L., Belikoff B., Buras J., Simms B.T., Mizgerd J.P., Carmeliet P., Karumanchi S.A., & Aird W.C. (2006). Vascular endothelial growth factor is an important determinant of sepsis morbidity and mortality. Journal of Experimental Medicine, 203, 1447-1458. https://doi.org/10.1084/jem.20060375

34. Yano, K., Okada Y., Beldi G., Shih S.C., Bodyak N., Okada H., Kang P.M., Luscinskas W., Robson S.C., Carmeliet P., Karumanchi S.A., & Aird W.C. (2008). Elevated levels of placental growth factor represent an adaptive host response in sepsis. Journal of Experimental Medicine, 205, 2623-2631. https://doi.org/10.1084/jem.20080398

35. Yoon, S.K. (2018). Oleuropein as an Antioxidant and Liver Protect. Oxidative Stress and Dietary Antioxidants. Amsterdam, The Netherlands: Elsevier Inc, 323-335.

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Research Article

Detection of Methicillin-resistant *Staphylococcus aureus* (MRSA) resistant to vancomycin and linezolid in bulk tank milk by E-test method

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ABSTRACT

Staphylococcus aureus is considered a serious threat to public health, besides is one of the most common causes of subclinical mastitis in dairy cows. Therefore, especially methicillin-resistant *Staphylococcus aureus* (MRSA) is among the most critical reasons for antibiotic treatment. Monitoring the antibiotic resistance of MRSA from livestock animals and foods is of great significance. This study aimed to detect vancomycin, teicoplanin, and linezolid resistance of bulk tank milk-borne 34 MRSA isolates by the E-test method to determine MIC values. In the study, it was determined that 8.8% of MRSA isolates were also resistant to vancomycin, and 11.7% to linezolid, while none of the isolates were determined to be resistant to teicoplanin. Data from the study reveal the status of the efficacy of the commonly used antibiotics vancomycin and linezolid against MRSA infections. Especially before MRSA treatment, MIC values of antibiotics should be determined, and appropriate antibiotics should be used in effective doses.

INTRODUCTION

Staphylococcus aureus is a ubiquitous, opportunistic, and commensal pathogen that may result in both community-acquired or nosocomial infections (Kadariya et al., 2014). On the other hand, *S. aureus* is one of the most common causes of subclinical mastitis in dairy cows and, therefore one of the most common causes of antibiotic treatment (Bouzidi et al., 2023). Nonetheless, methicillin-resistant *Staphylococcus aureus* (MRSA) is considered a critical threat, with 10,600 deaths and 323,700 cases per year, according to Antibiotic Resistance Threats 2019 report in the US (Centers for Disease Control and Prevention, 2019). Therefore, the treatment of staphylococcal infections is of great importance in terms of public health.

MRSA is often analyzed from food and livestock samples (Aires-de-Sousa et al., 2017; Sergelidis and Angelidis, 2017). Besides their resistance to almost all beta-lactam antibiotics, resistance to vancomycin and linezolid, which are considered last resort antibiotics and therefore critical, has become a significant concern for human and animal health for reducing treatment choices of severe infections caused by MRSA (Onaran et al., 2019; Mamfe et al., 2021).

Over the years, the common use of vancomycin in healthcare institutions has led to the emergence of glycopeptide-resistant strains of *S. aureus*. Vancomycin-resistant *S. aureus* (VRSA) cases are increasing daily; this situation is considered an important threat to public health. Following the first report from Japan in 1997, VRSA has been reported in various countries (Adegoke et al., 2014). However, most of these reports are related to hospitalized human patients with pre-existing MRSA infections (Lienen et al., 2022).

Linezolid-resistant *S. aureus* (LRSA) strains in humans, livestock, and food have been reported in studies; however, the prevalence of resistance was generally lower compared to other antibiotics (Timmermans et al., 2021). In one health context, the handling of livestock or food has been indicated as a risk factor for livestock-associated MRSA infections in animals (George et al., 2017). Therefore, it should be taken into account that the ingestion of LRSA from livestock animals can lead to antibiotic-resistant infections in people related to the food or livestock industry. In conclusion, monitoring antibiotic resistance in MRSA from livestock and food is highly significant (Lienen et al., 2022).

The E-test method is a suitable option because it is an easy-to-apply and quick-result analysis used to determine whether an isolate is antibiotic-resistant by detecting the Minimal Inhibition Concentration (MIC) value of the determined antibiotic. The results are also easy to interpret. For the reasons mentioned above, it has been suggested to use the E-test method as a routine test to determine the MIC values of antibiotics (Tandel et al., 2012; Phillips et al., 2016). For this reason, this study aimed to detect vancomycin, teicoplanin, and linezolid resistance of milk-borne MRSA isolates with subclinical mastitis by the E-test method.

MATERIAL AND METHODS

Bacterial isolates

The current study used 34 MRSA isolates that were previously isolated in a study conducted by Keyvan et al. (2020).

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Briefly, bulk tank milk samples were subjected to plating on a medium known as rabbit plasma fibrinogen agar (BP-RPF, Oxoid, Italy) and subsequently incubated at a temperature of 37°C for a duration of 24-48 hours. The isolates verified by PCR analysis with primer pairs of species-specific *nuc*, *coa,* and *mecA* genes were used.

Minimal Inhibition Concentration values

The resistance profiles of these isolates against vancomycin, teicoplanin, and linezolid antibiotics were investigated using the E-test method to determine MIC values. Using the swab technique, bacterial suspensions of a 0.5 McFarland standard inoculum in BHI (Brain Heart Infusion) broth (Merck 110493, Darmstadt, Germany) were spread on Mueller Hinton Agar (Oxoid CM0337, Dublin, Ireland) plates. Vancomycin and teicoplanin MIC values of the isolates were determined using Himedia EM111-60ST (Maharashtra, India), and linezolid MIC values were determined using Himedia EM029-60ST (Maharashtra, India) E-test strips. E-test strips were placed onto the agar plates and incubated at 35°C for 24-48 h. After incubation, isolates were categorized as susceptible, intermediate, or resistant to related antibiotics considering the breakpoints stated by the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2023).

According to EUCAST breakpoint tables for interpretation of MICs and zone diameters version 13.0, 2023, for *S. aureus*, breakpoints for teicoplanin and vancomycin is 2 µg/ml and the linezolid breakpoint is 4 µg/ml. In other words, isolates with vancomycin and teicoplanin MIC values above 2 µg/ml were evaluated as vancomycin and teicoplanin-resistant isolates, and linezolid MIC values above 4 µg/ml were evaluated as linezolid-resistant *S. aureus* isolates (European Committee on Antimicrobial Susceptibility Testing, 2023).

On the other hand, according to CLSI (2023), breakpoint table M100, 33 rd. edition, isolates with vancomycin MIC values of 4-8 µg/ml were considered intermediate, and those with 16 µg/ml and above were considered resistant; teicoplanin MIC values of 32 µg/ml and above were considered resistant, and those with 16 µg/ml were considered intermediate; linezolid MIC values of 8 µg/ml and above were considered resistant (Clinical and Laboratory Standards Institute, 2020).

RESULTS

The vancomycin, teicoplanin, and linezolid MIC values of the isolates are given in Table 1.

According to EUCAST MIC values, three of the MRSA isolates were determined as vancomycin-resistant and four as linezolid-resistant, but none of the isolates were determined as teicoplanin resistant. In addition, one of the isolates was found resistant to both vancomycin and linezolid. By the MIC values reported by CLSI, one of the isolates was intermediate resistant to vancomycin, and two isolates were resistant to linezolid, while none of the isolates were analyzed to be resistant to teicoplanin. The numbers of isolates defined as vancomycin, teicoplanin, and linezolid resistant, intermediate, and susceptible according to EUCAST and CLSI are given in Table 2.

DISCUSSION

Antibiotic resistance has increased among various pathogens and the risk of transmission of resistant microorganisms to humans, as well as the ineffectiveness of current antibiotic therapy, has become a critical public health concern (Campos et al., 2022). In the livestock industry, subclinical mastitis causes problems related to the use of antibiotics and hence economic losses not only in Turkey but also worldwide (Vanderhaeghen et al., 2010). Although herd management programs can help reduce the number of clinical cases, *S. aureus*-related mastitis is one of the leading causes of bovine mastitis. Moreover, MRSA, which causes nosocomial infections and high mortality in humans, has been frequently isolated from subclinical mastitis cases in recent years (Bouzidi et al., 2023; Algammal et al., 2020).

Food can also be contaminated with MRSA at various stages of food processing from food-producing animals or by infected food industry workers (Al-Amery et al., 2019) such as the incidence of subclinical mastitis in dairy farming, mistreatment of animals, and poor sanitation conditions. Treatment failure is due to the ineffective use of antibiotics, the emergence of multidrug-resistant pathogens, and chronic infections with fibrosis (Seegers and Fourichon, 2003; Keyvan, 2023).

The study determined that 8.8% of MRSA isolates isolated from milk with subclinical mastitis were also resistant to vancomycin and 11.7% to linezolid, according to EUCAST. Data from the study reveal the status of the efficacy of the commonly used antibiotics vancomycin and linezolid against MRSA infections.

Vancomycin is widely used for the treatment of serious MRSA infections. To date, intermediate or resistant to vancomycin (MIC>2 µg/ml) animal-origin MRSA isolates are rarely encountered (Al-Amery et al., 2019). However, MRSA isolates with vancomycin MICs at the upper end of the sensitive range (MIC=1.5 to 2.0 µg/ml), which constitute the majority (82.3%) of our isolates, are also more common, especially in patients who received prior treatment with vancomycin. Vancomycin MICs of 1.5 and 2.0 µg/ml are associated with the ineffectiveness of vancomycin treatment (Maor et al., 2009; Soriano et al., 2008). It should be noted that contamination of the environment with VRSA in human health facilities leads to further colonization of food-producing animals (Charlton et al., 2014). The study conducted by Ozturk et al. (2019) demonstrated that *S. aureus* strains isolated from goat milk in a similar region of Turkey showed sensitivity to vancomycin.

The isolates with vancomycin MIC values of ≤2 µg/ml used in the study were reported susceptible according to EUCAST and CLSI breakpoints. However, for some MIC values, there is a discrepancy between the relevant guidelines regarding the identification of resistant and susceptible isolates. EUCAST breakpoints state that MICs>2 µg/ml should be reported as resistant (European Committee on Antimicrobial Susceptibility Testing, 2023), while CLSI considers MICs of 4–8 µg/ml to be moderate and those ≥16 µg/ml as resistant (Clinical and Laboratory Standards Institute, 2020). The resistance status of isolates varies depending on which guidelines are taken

Table 1. Vancomycin, teicoplanin, and linezolid MIC values of the MRSA isolates (µg/ml)

Sample Code	VAN	TEI	LIN
8b	2	0,5	3
10b	1,5	1	0,75
11a	2	1	1
15a	1,5	0,5	2
16	1,5	0,5	3
17b	1,5	<0,5	4
19a	1,5	0,75	2
20a	2	<0,5	2
25b	4	0,5	8
26a	3	0,5	2
26b	1,5	0,5	3
29a	1,5	0,5	6
29b	2	0,5	4
31a	1,5	<0,5	<0,5
31b	2	0,5	1,5
32b	1,5	0,75	8
38a	1,5	0,75	0,75
39a	1,5	0,5	3
41a	1,5	0,75	2
46b	2	0,5	2
48a	1,5	0,75	3
57	1,5	0,75	4
62	2	0,75	3
65	1	1	2
89b	1	0,5	2
99	1,5	<0,5	2
100	3	<0,5	3
101b	1,5	<0,5	6
102a	2	0,75	4
104b	2	0,5	3
107a	2	0,75	2
109a	2	0,75	2
115b	2	0,5	3
120b	1	0,5	2

VAN: vancomycin, TEI: teicoplanin, LIN: linezolid

Table 2. Number of isolates defined as resistant, intermediate, and susceptible to vancomycin, teicoplanin, and linezolid, according to EUCAST (2023) and CLSI (2023).

Number of isolates	VAN			TEI			LIN		
	R	I	S	R	I	S	R	I	S
EUCAST	3	-	31	-	-	34	4	-	30
CLSI	-	1	33	-	-	34	2	-	32

VAN: vancomycin, TEI: teicoplanin, LIN: linezolid; R: Resistant; I: Intermediate; S: Susceptible

as a basis. To cite an example from our study, only one isolate (2.9%) in the study was intermediate for CLSI, while 3 (8.8%) were resistant to vancomycin for EUCAST guidelines. As a striking detail from the results, the intermediate-resistant isolate was also completely resistant to linezolid according to both EUCAST and CLSI. In addition, 5.8% (2/34) of the isolates, according to CLSI, and 11.7% (4/34) of the isolates according to EUCAST were resistant to linezolid. Therefore, when determining vancomycin resistance, the MIC value of the isolate or the guideline for which the resistance was determined should be specified.

The use of vancomycin in the treatment of MRSA infections is becoming more and more suspicious, especially as reports of decreased susceptibility of isolates become more common (Al-Amery et al., 2019; Charlton et al., 2014). On the other hand, various studies had highlighted the association of vancomycin with nephrotoxicity, which is more common in higher doses (≥4 g/d) in treatment for *S. aureus* (Stokes, 2017). In light of the data obtained from our study, it can be said that the use of linezolid in MRSA infections has become useless.

Determination of MIC values is frequently used in making treatment decisions, especially to determine the decreased vancomycin susceptibility in MRSA isolates and to increase treatment efficiency. Alternative treatments to vancomycin are limited in MRSA infections. Linezolid is frequently used in the treatment of MRSA. However, the increasing number of LRSA isolates in studies from both isolates from hospital-acquired infections and isolates from food-producing animals makes the efficacy of this treatment problematic (Leao et al., 2022; Lienen et al., 2022). The results of our study also support this data.

It has been shown that daptomycin is effective in treating MRSA bacteremia, except when caused by left-sided endocarditis, and can maintain bactericidal activity despite slightly elevated MICs (Cui et al., 2006; Humphries et al., 2013). However, the use of daptomycin is limited because it is expensive and not routinely used. It has also been reported that some MRSA isolates develop daptomycin and vancomycin resistance in parallel (Humphries et al., 2013). A recent study reported that fosfomycin showed potent antimicrobial activity against MRSA isolates with resistance or reduced activity to other anti-MRSA antibiotics, including vancomycin, linezolid, and daptomycin (Saravolatz and Pawlak, 2023).

It has been widely reported that teicoplanin, which has a lipoglycopeptide structure similar to vancomycin in terms of its mechanism of action and efficacy, has fewer side effects than vancomycin (Svetitsky et al., 2009). However, studies on the dose of teicoplanin that should be used to effectively treat MRSA infections are limited. Studies conducted within this scope emphasized that the use of higher teicoplanin maintenance dose is very important especially in severe infections due to MRSA (Lee et al., 2022).

CONCLUSION

In our study, all MRSA isolates were sensitive to teicoplanin, and similar studies on clinical and food-borne isolates also found complete sensitivity to teicoplanin (Sukri et al., 2023; Yucel et al., 2011). Concomitant susceptibility to linezolid, teicoplanin, and/or vancomycin in clinical and food-borne isolates in the aforementioned studies is remarkable and shows different results from our study. For these reasons, the determination of antibiotic MICs to determine antimicrobial susceptibility is of critical importance internationally.

DECLARATİONS

Ethics Approval: Ethics committee approval is not required since humans/animals were not used in our study.

Conflict of Interest: Authors do not have any conflict of interest to disclose nor do they endorse the use of any product/technology/service over the other.

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Author contribution

Idea, concept and design: BO, EK

Data collection and analysis: BO, EK

Drafting of the manuscript: BO, EK

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REFERENCES

1. Adegoke, A. A., & Okoh, A. I. (2014). Species diversity and antibiotic resistance properties of *Staphylococcus* of farm animal origin in Nkonkobe Municipality, South Africa. Folia Microbiologica, 59, 133-140. https://doi.org/10.1007/s12223-013-0275-1

2. Al-Amery, K., Elhariri, M., Elsayed, A., El-Moghazy, G., Elhelw, R., El-Mahallawy, H., El Hariri, M., & Hamza, D. (2019). Vancomycin-resistant *Staphylococcus aureus* isolated from camel meat and slaughterhouse workers in Egypt. Antimicrobial Resistance & Infection Control, 8, 1-8. https://doi.org/10.1186/s13756-019-0585-4

3. Aires-de-Sousa, M. (2017). Methicillin-resistant *Staphylococcus aureus* among animals: current overview. Clinical Microbiology and Infection, 23(6), 373-380. https://doi.org/10.1016/j.cmi.2016.11.002

4. Algammal, A. M., Enany, M. E., El-Tarabili, R. M., Ghobashy, M. O., & Helmy, Y. A. (2020). Prevalence, antimicrobial resistance profiles, virulence and enterotoxins-determinant genes of MRSA isolated from subclinical bovine mastitis in Egypt. Pathogens, 9(5), 362. https://doi.org/10.3390/pathogens9050362

5. Bouzidi, S., Bourabah, A., Cheriet, S., Abbassi, M. S., Meliani, S., & Bouzidi, H. (2023). Occurrence of virulence genes and methicillin-resistance in *Staphylococcus aureus* isolates causing subclinical bovine mastitis in Tiaret area, Algeria. Letters in Applied Microbiology. ovad003, https://doi.org/10.1093/

lambio/ovad003

6. Campos, B., Pickering, A. C., Rocha, L. S., Aguilar, A. P., Fabres-Klein, M. H., de Oliveira Mendes, T. A., Fitzgerald, J. R. & & de Oliveira Barros Ribon, A. (2022). Diversity and pathogenesis of *Staphylococcus aureus* from bovine mastitis: current understanding and future perspectives. BMC Veterinary Research, 18(1), 1-16. https://doi.org/10.1186/s12917-022-03197-5

7. Centers for Disease Control and Prevention (2019). 2019 AR Threats Report. https://www.cdc.gov/drugresistance/biggest-threats.html

8. Charlton, C. L., Hindler, J. A., Turnidge, J., & Humphries, R. M. (2014). Precision of vancomycin and daptomycin MICs for methicillin-resistant *Staphylococcus aureus* and effect of subculture and storage. Journal of Clinical Microbiology, 52(11), 3898-3905. https://doi.org/10.1128/JCM.01571-14

9. Clinical and Laboratory Standards Institute. (2023). M100. Performance standards for antimicrobial susceptibility testing, 33rd edition. https://clsi.org/standards/products/microbiology/documents/m100/

10. Cui, L., Tominaga, E., Neoh, H. M., & Hiramatsu, K. (2006). Correlation between reduced daptomycin susceptibility and vancomycin resistance in vancomycin-intermediate *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy, 50(3), 1079-1082. https://doi.org/10.1128/AAC.50.3.1079-1082.2006

11. European Committee on Antimicrobial Susceptibility Testing. (2023). According to EUCAST breakpoint tables for interpretation of MICs and zone diameters version 13.0, 2023. https://www.eucast.org/clinical_breakpoints

12. Goerge, T., Lorenz, M. B., van Alen, S., Hübner, N. O., Becker, K., & Köck, R. (2017). MRSA colonization and infection among persons with occupational livestock exposure in Europe: prevalence, preventive options and evidence. Veterinary Microbiology, 200, 6-12. https://doi.org/10.1016/j.vetmic.2015.10.027

13. Humphries, R. M., Pollett, S., & Sakoulas, G. (2013). A current perspective on daptomycin for the clinical microbiologist. Clinical microbiology reviews, 26(4), 759-780. https://doi.org/10.1128/CMR.00030-13

14. Kadariya, J., Smith, T. C., & Thapaliya, D. (2014). *Staphylococcus aureus* and staphylococcal food-borne disease: an ongoing challenge in public health. BioMed Research International, 2014. Article ID 827965. https://doi.org/10.1155/2014/827965

15. Keyvan, N. (2023). Suda Çözünen Vitaminler ve Özellikleri. In: H. Y. Cogun (Ed.) Veteriner Hekimliği Bilimlerinde Güncel Tartışmalar 2 (pp. 159-169). Bilgin Kültür Sanat Yayınları

16. Keyvan, E., Yurdakul, O., Demirtas, A., Yalcin, H., & Bilgen, N. (2020). Identification of methicillin-resistant *Staphylococcus aureus* in bulk tank milk. Food Science and Technology, 40(1), 150-156. https://doi.org/10.1590/fst.35818

17. Leão, C., Clemente, L., Cara d'Anjo, M., Albuquerque, T., & Amaro, A. (2022). Emergence of *cfr*-mediated linezolid resistance among livestock-associated Methicillin-Resistant *Staphylococcus aureus* (LA-MRSA) from Healthy Pigs in Portugal. Antibiotics, 11(10), 1439. https://doi.org/10.3390/antibiotics11101439

18. Lee, J. H., Choi, M. G., Park, H. J., Kim, H. C., & Choi, C. M. (2022). Comparison of mortality and clinical failure rates between vancomycin and teicoplanin in patients with methicillin-resistant *Staphylococcus aureus* pneumonia. BMC Infectious Diseases, 22(1), 1-10. https://doi.org/10.1186/s12879-022-07549-2

19. Lienen, T., Grobbel, M., Tenhagen, B. A., & Maurischat, S. (2022). Plasmid-Coded Linezolid Resistance in Methicillin-Resistant *Staphylococcus aureus* from Food and Livestock in Germany. Antibiotics, 11(12), 1802. https://doi.org/10.3390/antibiotics11121802

20. Mamfe, L. M., Akwuobu, C. A., & Ngbede, E. O. (2021). Phenotypic detection, antimicrobial susceptibility and virulence profile of staphylococci in the pig production setting, Makurdi, Nigeria. Access Microbiology, 3(12), 293. https://doi.org/10.1099/acmi.0.000293

21. Maor, Y., Hagin, M., Belausov, N., Keller, N., Ben-David, D., & Rahav, G. (2009). Clinical features of heteroresistant vancomycin-intermediate *Staphylococcus aureus* bacteremia versus those of methicillin-resistant *S. aureus* bacteremia. The Journal of Infectious Diseases, 199(5), 619-624. https://doi.org/10.1086/596629

22. Onaran, B., Göncüoğlu, M., Ormancı, F. S. B. (2019). Antibiotic resistance profiles of vancomycin resistant enterococci in chicken meat samples. Ankara Üniversitesi Veteriner Fakültesi Dergisi, 66(4), 331-336. https://doi.org/10.33988/auvfd.451328

23. Ozturk, D., Turutoglu, H., Pehlivanoglu, F., & Yapicier, O. S. (2019). Identification of bacteria isolated from dairy goats with subclinical mastitis and investigation of methicillin and vancomycin resistant *Staphylococcus aureus* strains. Ankara Üniversitesi Veteriner Fakültesi Dergisi, 66(2), 191-196. https://doi.org/10.33988/auvfd.431465

24. Phillips, C. J., Wells, N. A., Martinello, M., Smith, S., Woodman, R. J., & Gordon, D. L. (2016). Optimizing the detection of methicillin-resistant *Staphylococcus aureus* with elevated vancomycin minimum inhibitory concentrations within the susceptible range. Infection and drug resistance, 9, 87-92. https://doi.org/10.2147/IDR.S107961

25. Saravolatz, L. D., & Pawlak, J. (2023). In vitro activity of fosfomycin alone and combination against *Staphylococcus aureus* with reduced susceptibility or resistance to methicillin, vancomycin, daptomycin or linezolid. Journal of Antimicrobial Chemotherapy, 78(1), 238-241. https://doi.org/10.1093/jac/dkac380

26. Seegers, H., Fourichon, C., & Beaudeau, F. (2003). Pro-

duction effects related to mastitis and mastitis economics in dairy cattle herds. Veterinary research, 34(5), 475-491. https://doi.org/10.1051/vetres:2003027

27. Sergelidis, D., & Angelidis, A. S. (2017). Methicillin□ resistant *Staphylococcus aureus*: a controversial food□ borne pathogen. Letters in applied microbiology, 64(6), 409-418. https://doi.org/10.1111/lam.12735

28. Soriano, A., Marco, F., Martínez, J. A., Pisos, E., Almela, M., Dimova, V. P., ... & Mensa, J. (2008). Influence of vancomycin minimum inhibitory concentration on the treatment of methicillin-resistant *Staphylococcus aureus* bacteremia. Clinical Infectious Diseases, 46(2), 193-200. https://doi.org/10.1086/524667

29. Stokes, M. B. (2017). Vancomycin in the kidney—a novel cast nephropathy. Journal of the American Society of Nephrology, 28(6), 1669-1670. https://doi.org/10.1681/ASN.2017010091

30. Sukri, K. M., Mohamed, N. A., & Ilina Isahak, A. A. M. (2023). Prevalence and Susceptibility of *Staphylococcus aureus* Nasal Carriage Strains Isolated from Haemodialysis Patients. Malaysian Journal of Medicine and Health Sciences 19(1), 181-187. https://doi.org/10.47836/mjmhs19.1.25

31. Svetitsky, S., Leibovici, L., & Paul, M. (2009). Comparative efficacy and safety of vancomycin versus teicoplanin: systematic review and meta-analysis. Antimicrobial Agents and Chemotherapy, 53(10), 4069-4079. https://doi.org/10.1128/AAC.00341-09

32. Tandel, K., Praharaj, A. K., & Kumar, S. (2012). Differences in vancomycin MIC among MRSA isolates by agar dilution and E test method. Indian Journal of Medical Microbiology, 30(4), 453-455. https://doi.org/10.4103/0255-0857.103768

33. Timmermans, M., Bogaerts, B., Vanneste, K., De Keersmaecker, S. C., Roosens, N. H., Kowalewicz, C., Simon, G., Argudín, M. A., Deplano, A., Hallin, M., Wattiau, P., Fretin, D., Denis, O., & Boland, C. (2022). Large diversity of linezolid-resistant isolates discovered in food-producing animals through linezolid selective monitoring in Belgium in 2019. Journal of Antimicrobial Chemotherapy, 77(1), 49-57. https://doi.org/10.1093/jac/dkab376

34. Vanderhaeghen, W., Cerpentier, T., Adriaensen, C., Vicca, J., Hermans, K., & Butaye, P. (2010). Methicillin-resistant *Staphylococcus aureus* (MRSA) ST398 associated with clinical and subclinical mastitis in Belgian cows. Veterinary microbiology, 144(1-2), 166-171. https://doi.org/10.1016/j.vetmic.2009.12.044

35. Yucel, N., Citak, S., & Bayhün, S. (2011). Antimicrobial resistance profile of *Staphylococcus aureus* isolated from clinical samples and foods of animal origin. Foodborne pathogens and disease, 8(3), 427-431. https://doi.org/10.1089/fpd.2010.0707

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Research Article

Morphological investigation of the veins and bile vessels of rabbit liver

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ABSTRACT

The focus was to investigate the anatomical specifics of v. portae, vv. hepaticae and ductus choledochus by corrosion. We investigated 10 sexually mature, clinically healthy New Zealand White rabbits, 8 months old, weighing 2.8 kg to 3.2 kg. To determine the veins and bile vessels, a cold-curing acrylic-based plastic (Duracryl +) was used. The main portal vessel was an intra-organic continuation of v. portae, after its branching into caudate lobe. The main portal vein was divided into v. portae dextra and v. portae sinistra, when entering lobus hepatis dexter and lobus hepatis sinister. V. portae sinistra caudalis was a branch of v. portae sinistra. The venous drainage of the rabbit liver in was carried out by v. hepatica sinistra caudalis, v. hepatica sinistra, v. hepatica dextra, v. hepatica media and venous vessel in lobus caudatus. V. hepatica sinistra and v. hepatica media had a common origin and took blood from lobus hepatis sinister medialis. V. hepatica dextra drained lobus hepatis dexter. V. hepatica sinistra caudalis was a direct tributary of the caudal vena cava. Ductus hepaticus communis was well developed and collected the bile from the main bile duct. Ductus hepaticus dexter drained lobus hepatis dexter and evacuated the bile into the main bile duct. Ductus hepaticus sinister caudalis flowed directly into ductus hepaticus communis. Ductus hepaticus sinister passed into the main bile duct.

INTRODUCTION

The anatomical features of the liver vessels in New Zealand white rabbits are similar to those in humans. Therefore, this species has been used as an anatomical model to study portal and hepatic veins in humans (Mapara et al., 2012).

Corrosion anatomical study of the liver in carnivores and humans is an appropriate method for studying variations in the morphological features of the liver vessels (Matusz, et al., 2007; Uršič et al., 2007).

According to De Graaf et al. (2011) the rabbit liver consists of four parts. The three cranial lobes are lobus hepatis dexter, lobus hepatis sinister medialis and lobus hepatis sinister lateralis, and the caudal lobe is lobus caudatus. In each lobe the portal vein sends branches.

Specific to the rabbit□s liver is that the cranial lobes are separated from the caudate lobe. The portal vein in the rabbit at the entrance to the liver is divided into a branch for the lobus caudatus and a main porta vessel. The main portal vein is subdivided into v. portae sinistra and v. portae dextra. V. portae sinistra sends a medial and lateral branch to lobus hepatis sinister medialis and lobus hepatis sinister lateralis. V. portae sinistra inferior is defined as an additional branch starting from the left portal or from the main portal vein (Seo et al. 2001; Páramo et al., 2017).

According to Barone (1997) and Barone (2011), the portal vein in the rabbit has two branches - left and right. The left branch supplies blood to lobus hepatis sinister medialis, lobus hepatis sinister lateralis and lobus quadratus, and the right branch – to lobus hepatis dexter.

The data from the corrosion method of the study of portal vessels in humans are used as a morphological basis for the interpretation of anatomical findings obtained in selective portography (Sinelnikov, 1973).

In the mole, eight hepatic veins have been described, which drain blood from the liver in v. cava caudalis. Proc. caudatus and lobus hepatis dexter lateralis have separate venous drainage from two separate hepatic veins. They unite in a common vein, which is an inflow of the caudal vena cava. Lobus hepatis dexter medialis, proc. papillaris and lobus quadratus are drained by three hepatic veins, which are also tributaries of the caudal vena cava. In the left hepatic lobe are found four vv. hepaticae (Nešić1 et al., 2020).

According to Carlisle et al. (1995) each hepatic lobe in the dog has a separate hepatic vein. The following hepatic veins are distinguished: left lateral and left medial vein, hepatic vein in lobus quadratus, right lateral and right medial vein, hepatic vein in proc. papillaris and hepatic vein in proc. caudatus.

According to Mari and Acocella (2015) ,the dog□s liver is divided into two sections, three subsections, seven sections and two to four subsections. The right section is drained by separate hepatic veins, while most of the left section is drained

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by the main hepatic vein formed by the fusion between the middle and left hepatic veins. Part of lobus hepatis dexter and proc. papillaris is drained directly by v. cava caudalis. The right hepatic vein drains only lobus hepatis dexter.

The venographic presentation of vv. hepaticae in the rabbit visualizes five separate vessels. Lobus caudatus has a separate venous drainage. V. hepatica sisnistra caudalis is a direct branch of caudal vena cava and determines the independent venous drainage of the left lateral hepatic lobe (Seo et al., 2001; Stamatova-Yovcheva, 2016; Stamatova-Yovcheva et al., 2018).

According to Barone (2011), ductus hepaticus communis is absent in the rabbit. The left bile duct drains lobus hepatis sinister medaialis, lobus hepatis sinister lateralis and lobus quadratus. The right bile duct drains lobus hepatis dexter and lobus caudatus. Ductus cysticus and ductus hepaticus sinister form ductus choledochus, and ductus hepaticus dexter joins ductus choledochus.

Alloush (1997) presented data on the presence of a separate ductus hepaticus communis in the rabbit, formed by the fusion of the left and right bile ducts, ventrally from porta hepatis. Ductus hepaticus communis joins the bile duct in proc. caudatus, at its transition into lig. hepatoduodenale.

According to other authors (Stamatova-Yovcheva, 2016; Stamatova-Yovcheva et al., 2018), the liver of the rabbit has a well-defined ductus hepaticus communis. The main biliary duct is a continuation of ductus hepaticus communis. Ductus hepaticus sinister caudalis is an independent direct branch of the common biliary duct and is located in lobus hepatis sinister lateralis. Ductus hepaticus sinister and ductus hepaticus dexter form the main biliary duct.

Sinelnikov (1973) describes the biliary tree in humans through comparative corrosion and contrast anatomical analysis, establishing compliance.

From the presented data it is evident that the anatomical results for the liver of the rabbit are contradictory and insufficient. Corrosion anatomical studies of this organ complement the findings of previous studies. Therefore, the reason for the present study is the expected results for the interpretation of the morphological features of the venous and bile vessels in the rabbit.

MATERIAL and METHODS

The study included 10 sexually mature, clinically healthy rabbits, 8 months old, of the New Zealand White breed, weighing 2.8 kg to 3.2 kg. In the studied animals, dissection was performed according to the algorithm described by Bensley (1948), Wingerd (1985), Yonkova, (2014) and Stamatova-Yovcheva (2016) for the rabbit.

Preparation of corrosion macroscopic preparations of v. portae and its branches we cannulated the portal vein, caudally from the pancreas. V. portae was ligated into the Th13-L1 segment. The carcasses were dissected in the segment from Th13 to L4. We applied an injection in v. portae of cold-curing acrylic-based plastic (Duracryl +, two-component SpofaDental, Chech Republic), according to the following prescription: -10 g purple colorant (TS, DEUTEK S.A.) was added to 30 g of the powdered component Duracryl +, two component, 30 g of hardener Duracryl + was added to the mixture. We injected 10 mL of the resulting homogenized solution into the cannula using a syringe. We placed the carcass segments at a refrigeration temperature (+ 4C °) for 24 hours, then moved them to a polyvinyl container with a lattice bottom. In a PVC vessel with a capacity of 7 L to 1.5 L of water we added 3 L of hydrochloric acid (38% h. HCL, MARVIN Ltd., Dimitrovgrad) (ratio 1: 2). We placed the lattice vessel in the vessel containing hydrochloric acid and water for a period of 48 hours. The corrosion preparations were washed in a weak water stream. The obtained results were photo-documented.

Preparation of corrosion macroscopic preparations from vv. hepaticae we investigated the localization of v. cava caudalis in the segment from Th10 to L3 in three rabbits. We cannulated the caudal vena cava at the L2 level. For greater clarity, we simultaneously filled with the cold polymer paste v. cava caudalis and ductus choledochus. For the injection of v. cava caudalis blue dye was used and for ductus choledochus – yellow dye.

Preparation of corrosion preparations from the gallbladder and bile vessels. We studied the topography of the liver, gallbladder, ductus choledochus, stomach and pars cranialis duodeni in four rabbits. A curved intestinal clamp was placed on pars cranialis duodeni, 20 mm caudally from the pylorus. The liver, stomach and pars cranialis duodeni were extirpated and placed in a 0.6 / 1.0 L Simax beaker (Czech Republic). The organs were washed under running water for 30 minutes. We applied a longitudinal incision on the antimesenteric wall of pars cranialis duodeni. Papilla duodeni major was cannulated by pyrogen-free cannula (PROBIO-silicone carbide, G18) with sequence number 18 in ductus choledochus. We used yellow colorant. We introduced 5 mL of the resulting homogenized solution into papilla duodeni major. The studied group of organs (liver, stomach and pars cranialis duodeni) were stored at refrigeration temperatures (+ 4C °) overnight. The rest of the research methodology coincides with the steps described for v. portae.

RESULTS

Corrosion anatomical study revealed that the main portal vessel was an intraorganic continuation of v. portae. It was divided into v. portae dextra and v. portae sinistra, when entering lobus hepatis dexter and lobus hepatis sinister. V. portae sinistra caudalis was a branch of v. portae sinistra in lobus hepatis sinister lateralis. The left portal vein was further divided into a lateral branch in lobus hepatis sinister lateralis and a medial branch in lobus hepatis sinister medialis. V. portae dextra was a single vessel in lobus hepatis dexter. The venous vessel, which was located dorsally from the portal vein, was a direct branch of v. portae and entered lobus caudatus (Figure 1 and Figure 2)

The venous drainage of the rabbit liver in was carried out by five hepatic veins: v. hepatica sinistra caudalis, v. hepatica sinistra, v. hepatica dextra, v. hepatica media and venous

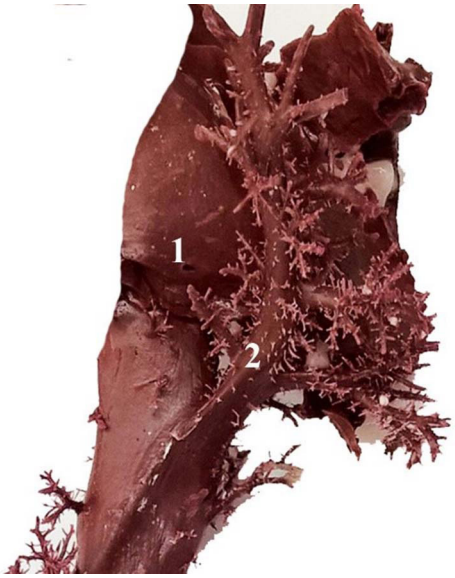

Figure 1. Corrosion anatomical image of a fragment of v. portae. (1) v. portae; (2) a branch of v. portae in lobus caudatus.

Figure 2. Corrosion anatomical image of a fragment of the main portal vessel and its branches in the rabbit liver. (1) main portal vessel; (2) v. portae dextra; (3) v. portae sinistra; (4) lateral branch of v. portae sinistra; (5) medial branch of v. portae sinistra (6) v. portae sinistra caudalis.

vessel in lobus caudatus. Vv. hepaticae in the rabbit did not have extraorganic areas and were visceral tributaries of v. cava caudalis. Lobus caudatus had a separate venous vessel that was a direct inflow of caudal vena cava (Figure 3).

Lobus hepatis sinister lateralis had a separate venous drainage, which was carried out by v. hepatica sinistra caudalis, a direct inflow of v. cava caudalis. The latter sent smaller branches in the dorsal, middle and ventral areas of the lateral left lobe. V. hepatica sinistra and v. hepatica media had a common origin and took blood from lobus hepatis sinister medialis. In lobus hepatis dexter was found v. hepatica dextra. V. hepatica media drained blood from lobus hepatis dexter and gallbladder (Figure 4).

Ductus hepaticus communis collected bile from the main bile duct. Ductus hepaticus dexter drained lobus hepatis dexter and evacuated the bile into the main bile duct. Ductus hepaticus sinister caudalis flowed directly into ductus hepaticus communis. Ductus hepaticus sinister passed into the main bile duct between lobus hepatis sinister medialis and lobus hepatis dexter. In lobus hepatis sinister medialis, ductus hepaticus sinister received bile from medial and lateral branches. The medial branch drained lobus hepatis sinister medialis, and the lateral one drained lobus hepatis sinister medialis and lobus quadratus. Ductus cysticus originated from the gallbladder and

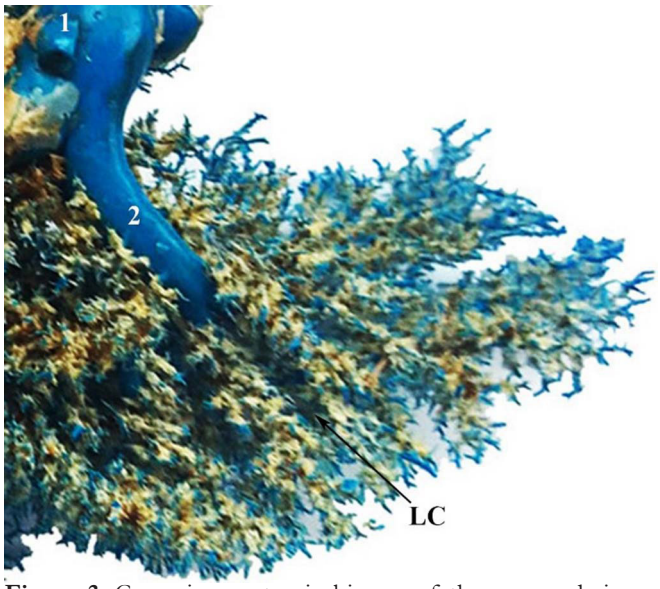

Figure 3. Corrosion anatomical image of the venous drainage in lobus caudatus in rabbit. (1) v. cava caudalis; (2) a venous vessel in lobus caudatus.

Figure 4. Corrosion anatomical image of fragment from rabbit liver. (1) v. cava caudalis; (2) v. hepatica sinistra caudalis; (3) v. hepatica sinistra; (4) v. hepatica media; (5) v. hepatica dextra; (6) vesica fellea; (7) v. cystica; LHSL – lobus hepatis sinister lateralis; LHSM-lobus hepatis sinister medialis; LHD – lobus hepatis dexter.

joined the main bile duct. Lobus caudatus had a separate bile drainage from a direct branch of ductus hepaticus communis (Figure 5).

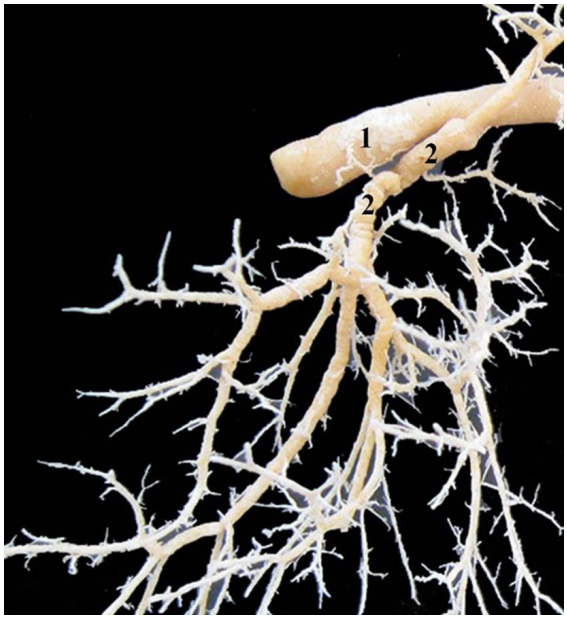

Figure 5. Corrosion anatomical image of fragment from ductus hepaticus communis in the rabbit. (1) ductus hepaticus communis; (2) ductus hepaticus sinister caudalis.

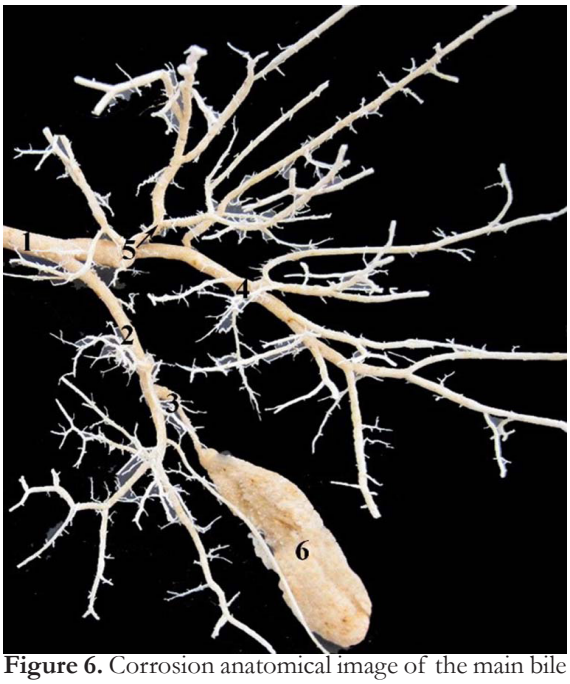

Figure 6. Corrosion anatomical image of the main bile duct in the rabbit (1) main bile duct; (2) ductus hepaticus sinister (3) ductus cysticus; (4) ductus hepaticus dexter; (5); bile duct in lobus caudatus (6) vesica fellea.

DISCUSSION

We present a modern view of the terminological justification of portal vessels in the rabbit. In our opinion, each unit has its own portal blood supply. Our hypothesis corresponds to the opinion of De Graaf et al. (2011) for the portal vascularization of the liver in the rabbit.

We consider that v. portae sinistra caudalis is a specific vessel for lobus hepatis sinister lateralis in the rabbit. In our opinion, the venous supply of lobus hepatis sinister medialis is carried out by v. portae sinistra medialis, the lateral branch of v. portae sinistra supplies lobus hepatis sinister lateralis, and

v. portae dextra supplies lobus hepatis dexter. Our hypothesis gives us reason to support the findings of Seo et al. (2001) and Páramo et al. (2017) on the morphology of the hepatic portal system in rabbits.

Our assertion is that the portal vessels in the rabbit are v. portae sinistra, v. portae sinistra caudalis, v. portae dextra and a portal vessel in lobus caudatus does not support the view of Barone (1997) and Barone (2011) that portal vascularization in the rabbit liver is performed only by v. portae dextra and v. portae sinistra.

We argue that the direct portal branch for lobus caudatus separates before the main portal vein and that the continuation of the portal vein after lobus caudatus is the main portal vessel that provides the branches for the remaining parts of the liver. Our thesis corresponds to previous research in this direction (Stamatova-Yovcheva, 2016; Stamatova-Yovcheva et al., 2018)., concerning the portal blood supply of the liver in the rabbit.

The found five hepatic veins the rabbit liver did not correspond to the describtion of Nešić et al. (2020) about the hepatic vascularization in the mole.

We argue that only lobus caudatus and lobus hepatis sinister lateralis have a separate venous drainage, which differs from the opinion of Carlisle et al. (1995) and Mari and Acocella (2015) for the venous drainage in the liver of the dog and complements the findings of some authors on the hepatic veins in rabbits (Seo et al., 2001; Stamatova-Yovcheva, 2016; Stamatova-Yovcheva et al., 2018).

The anatomical data obtained by us from the corrosion anatomical examination of the bile ducts are convincing enough and present information about the presence of ductus hepaticus communis in the rabbit. Our data differ from the thesis of Barone (2011), according to which ductus hepaticus communis in rabbits is missing and complements the published data on the morphological features of the bile ducts in rabbits (Alloush, 1997; Stamatova-Yovcheva, 2016; Stamatova-Yovcheva et al., 2018).

The results presented by us from the corrosion of the organ, which concern the course of the venous and bile vessels in the liver of the rabbit is a valid criterion for accepting the reliability of the method. Therefore, we can support the opinion of some authors (Matusz, et al., 2007; Uršič et al., 2007, Sinelnikov, 1973) about humans and carnivores, for the application of the corrosion method in the study of the liver in rabbits.

CONCLUSION

The corrosion anatomical study of the blood and bile vessels in the rabbit gives a detailed picture of the macroarchitectonics of these vessels. We propose that our results can be used as a morphological basis in the corrosion study of the hepatic blood and bile vessels in other mammals and humans.

DECLARATIONS

Ethics Approval

The experiments were conducted in strict compliance with the ethical guidelines of Trakia University (protocol 209/24.10.20 12;213/14.11.2012;220/12.12.2012; 231/04.02.2013).

Conflict o Interest

The authors declare that there have no conflict of interests.

Author Contributions

Idea, concept and design: KSY, RD, ÖG.

Data Collection and analysis: KSY, RD, DY.

Drafting of the manuscript: KSY, ÖGD.

Critical review: KSY, RD, ÖGD, DY.

REFERENCES

1. Alloush, G. M. (1997). Some anatomical studies of the gross morphological features of the liver of rabbit with special reference to its location pattern. Faculty of Veterinary Medicine, Cairo University, Egypt.

2. Barone R., (1997). Chapitre VII - Foie. In: Anatomie comparée des mammifères domestiques. Splanchnologie I. Tome troasième, Troisième edn, ed R. Barone, Vigot, Paris, pp. 507-560.

3. Barone, R. (2011). Veine cave caudale. In: Anatomie comparée des mammifères domestiques. Angiologie. Tome cinquième. Deuxième edn, ed R, Barone, Vigot, Paris, pp. 649-669.

4. Bensley B. (1948). Part III. Rabbit Dissection. IV. The Liver. In. Practical Anatomy of the Rabbit: An Elementary Laboratory Textbook in Mammalian Anatomy, ed B. Bensley, The University Press, Toronto, pp. 120-121.

5. Carlisle, C., J. Wu & Heath, T. (1995). Anatomy of the portal and hepatic veins of the dog: A basis for systematic evaluation of the liver by ultrasonography, Veterinary Radiology & Ultrasound, 36(3), 227-233, doi: 10.1111/j.1740-8261. 1995.tb00251. x.

6. De Graaf, W., Van den Esschert, J. W.,Van Lienden, K. P., Roelofs, J. J.İ & Van Gulik, T. M. (2011). A rabbit model for selective portal vein embolization, The Journal of Surgical Research, 171(2), 486–494, doi: 10.1016/j.jss.2010.04.032.

7. Mapara, M, Thomas B. S., Bhat, K. M. (2012). Rabbit as an animal model for experimental research. Dental Research Journal (Isfahan), 9(1), 111–118, doi: 10.4103/1735-3327.92960.

8. Mari, L. & Acocella, F. (2015). Vascular anatomy of canine hepatic venous system: A basis for liver surgery, Anatomia, Histologia, Embryologia, 44(3), 212-224, doi: 10.1111/ ahe.12129.

9. Matusz, P. L., Zahoi, D. E., Branzaniuc, K., Copotoiu, C., Stefan, C., Pusztai, A., Stefan, A. & E. Hordovan, E. (2007). The arterial vascularization of the liver's left medial division. Study on corrosion casts. The Federation of American Societies for Experimental Biology Journal, 21, 6. doi.org/10.1096/ fasebj.21.6. A968-b.

10. Nešić1, I., Krstić, N., Djelić, N, Zdravković, M., Tošković, B., Djordjević, M. & Blagojević, M. (2020). Folia Morphologica, 80(4), 888-894, doi: 10.5603/FM. a2020.0132.

11. Páramo, M., P., García-Barquin, E., Santa María, J., Miguel Madrid, M., Caballeros, A., Benito, B., Sangro, M., Iñarrairaegui, M., & Bilbao, J. I. (2017). Evaluation of the rabbit liver by direct portography and contrast-enhanced computed tomography: anatomical variations of the portal system and hepatic volume quantification. European Radiology Experimental, 1(7), 1-7. doi 10.1186/s41747-017-0011-8.

12. Seo, T., Oh, J., Lee, D., Ko, Y., & Yoon, Y. (2001). Radiologic anatomy of the rabbit liver on hepatic venography, arteriography, portography, and cholangiography, Investigative Radiology, 36(3), 186-192, doi: 10.1097/00004424-200103000-00008.

13. Sinelnikov, R. (1973). Liver. In: Atlas of Human Anatomy II, ed R. Sinelnikov, Medicine, Moscow, pp. 81-91.

14. Stamatova-Yovcheva K., Dimitrov R., Dilek, Ö.G. (2018). Radiographic study of the topography of the hepatic vasculature and bile ducts of the rabbit. Bulgarian Journal of Agricultural Science, 24(3), 497-502.

15. Stamatova-Yovcheva, K. (2016). The application of some imaging anatomical methods for investigation of the liver of the New Zealand white rabbit (Oryctolagus cuniculus). PhD thesis, Trakia University, Stara Zagora, Bulgaria

16. Uršič, M., Ravnik, D., Hribernik, M., Pečar, J., Butinar, J., Fazarinc, G. (2007). Gross Anatomy of the Portal Vein and Hepatic Artery Ramifications in Dogs: Corrosion Cast Study. Anatomia, Histologia, Embryologia, 36 (2), 83-87, doi: 10.1111/j.1439-0264.2006. 00719.x.

17. Wingerd, B., (1985). Principles & Techniques of Dissection. In: Rabbit Dissection Manual, ed B. Wingerd, The Johns Hopkins University Press, Baltimore, pp. 12-14.

18. Yonkova, P. (2014). Morphological studies on the mast depots of the New Zealand white rabbit. PhD thesis, Trakia University, Stara Zagora, Bulgaria.

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Research Article

Total phenolic content, antibacterial and antiradical properties of bee bread from Turkey

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ABSTRACT

Pollen grains, honey, and lactic acid bacteria are combined to make bee bread, which serves as the hive's one of the sources. This study aimed to evaluate a Turkish bee bread concerning the total phenolic content, antiradical, and antimicrobial activity against *Bacillus cereus, Escherichia coli* O157:H7, *Staphylococcus aureus*, and *Salmonella Typhimurium*. Folin-Ciocalteu method and DPPH test were used to determine the total phenolic content (TPC) and antiradical activity of the aqueous extract of bee bread, respectively. Antibacterial activity of the extract on the bacteria was evaluated using minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) methods. The TPC of the bee bread was found to be 24.45 ± 3.75 g GAE/mg. The DPPH assay results indicated that the water-soluble extract of bee bread (1 mg/mL) had a scavenging activity of 3.40±2.99%. Additionally, it showed an antibacterial effect on *S. aureus, E. coli, S. Typhimurium*, and *B. cereus* at the different concentrations (6.25 to 25 mg/mL). Overall, it was revealed that the bee bread had high total phenolic content and antiradical activity. Also, it showed antibacterial activity on all of the tested bacteria. This research contributes to the knowledge of the bioactive properties of this unexplored natural material.

INTRODUCTION

The species *Apis mellifera*, commonly known as honeybees, complete their dietary requirements by gathering nectar and pollen. The primary source of carbohydrates for bees is nectar, while pollen serves as a source of proteins, lipids, vitamins, and minerals. Bee bread refers to a blend of bee pollen, bee salivary enzymes, and honey or nectar that undergoes a process of fermentation within the honeycomb cells of a hive. The substance is utilized as sustenance for both the worker bees and the developing larvae (Sobral et al., 2017). Numerous studies of bee bread's chemical composition have shown that it usually contains water, protein, free amino acids, carbohydrates, fatty acids, minerals, vitamins, and many other bioactive compounds such as kaempferol, rutin, quercetin, luteolin, rosmarinic acid. The composition of bee bread varies depending on parameters such as, geographical area, the climatic conditions of the plants producing honey and seasonal changes (Mohammad et al., 2020; Bayram et al., 2021; Tutun et al., 2021; Ćirić et al., 2022). The chemical compositions that occur due to these conditions make bee bread a potential functional food with different bioactive components as well as being food source for bees (Bakour et al., 2022). Bee bread has a wide range of biological properties including antioxidant, antibacterial, antifungal, antiviral, anti-inflammatory, and anticancer (Didaras et al., 2020; Khalifa et al., 2020; Tutun et al., 2021). These biological properties are closely related to the chemical composition of bee bread. Bee bread is regarded as a dietary supplement owing to its biological impacts. In recent times, there has been a surge in interest regarding the utilization of diverse bee bread and its products for the treatment of numerous illnesses (Khalifa et al., 2020). Anatolia serves as a connecting link between three continents owing to its geographical location. The diverse flora and varying climatic conditions across different regions make it highly beneficial for beekeeping activities (Kambur and Kekeçoğlu, 2020). Turkey has 75% of the world's honey plants flora. Of the 11,500 species of flowering plants found in European countries, more than 9,000 are found in Turkey, of which 3,000 are endemic (Suna, 2019). Despite the significance of honey production among beekeepers in Turkey, the production of bee pollen and bee bread does not receive sufficient attention. In addition, studies on the content and biological activities of bee bread produced in different regions are very limited. This study aimed to determine the total phenolic content, antiradical, and antibacterial properties of bee bread collected from the Ankara province of Turkey.

MATERIAL and METHODS

Sampling and extraction

Samples of fresh bee bread were obtained from apiaries located in three different points of Ankara province, in the inner Anatolia region of Turkey. Samples were stored at 4 °C until testing. Aqueous extraction was carried out by adding 50 g of the homogenized sample to 200 mL of distilled water at room temperature and left in the dark for 2 days. Subse-

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quently, the samples were filtered through a filter paper, and the filtrates were concentrated at 40 °C in rotary evaporation (RV10, IKA®, Germany). The concentrated extracts received the process of evaporation and were subsequently subjected to drying in a freeze-dryer (Martin Christ, Alpha 1-2LD Plus, Germany) to obtain a crude extract. The lyophilized extract was stored in a tube at +4 °C in the dark for further analysis (Akhir et al., 2017).

Total phenolic content (TPC)

TPC was determined in aqueous extracts of bee bread by using the Folin–Ciocalteu method which was described by Pelka et al. (2021). The extract was dissolved in distilled water to obtain a concentration of 1 mg/mL. A volume of 50 µL of Folin-Ciocalteu reagent diluted (1:10, v/v) with high purity deionized water was added to a 96-well plate containing 10 µL of the extract solution. Following a 5-minute incubation period, 40 µL of Na_2CO_3 (7.5%) solution was added to the mixture. Following the stirring, 100 µL of high-purity water was added to the mixture and the volume reached 200 mL. The mixture was then allowed to incubate at room temperature for 30 minutes. The absorbance of the reaction was detected at 725 nm using a spectrophotometer (MultiskanGo, ThermoScientific). For constructing the calibration curve, fresh Gallic acid standard solutions (3.13 to 200.00 µg/mL) were prepared. Ethanol was used as a blank. The quantification of total phenolic content in the extracts was conducted by expressing the content as milligrams of Gallic acid equivalent (GAE) per gram of the extract. The measurements were conducted in duplicate (Pelka et al., 2021).

Antiradical activity

The radical scavenging activity of the extract was assessed by using (2,2-Diphenyl-1-picrylhydrazyl) assay according to the method reported by Kahraman et al. (2022). Briefly, a 50 µL of aqueous extract at a concentration of 1 mg/mL or ascorbic acid dissolved in ethanol (50 µg/mL) was mixed with 150 µL of a 200 µM methanolic DPPH solution in a 96-well plate. The mixture was incubated at room temperature for 30 minutes under dark conditions. Absolute methanol was used as blank. The results were measured at 517 nm in absorbance using a microplate reader (Multiskan Go, Thermo Scientific). The DPPH radical scavenging activity (%) was calculated as follows: DPPH scavenging activity (%) = [(Ac – As) / Ac] × 100, where Ac represented the absorbance of the control group [DPPH + Methanol without sample], As indicated the absorbance of the sample group [DPPH + Sample (extract)].

Antimicrobial activity

Bacterial strains

The strains of *Bacillus cereus*, *Escherichia coli* O157:H7, *Staphylococcus aureus* and *Salmonella* Typhimurium were obtained from the stock culture collection of Burdur Mehmet Akif Ersoy University's Department of Food Hygiene and Technology Laboratory. The bacterial strains were inoculated onto tryptic soy agar (TSA, Merck, Germany) and incubated at 37 °C for 18-24 h.

Microdilution method

The microdilution method was used to determine bee bread's minimal inhibitory concentration (MIC) values against *S. aureus* and *E. coli*, *S.* Typhimurium, and *B. cereus* (CLSI, 2006). The bacterial inoculums were prepared from the bacterial culture grown in TSA (Merck, Germany) at 37 °C for 18 h and adjusted to 0.5 McFarland (≈ 1.5 x 10^8 CFU/mL) in 0.9% sterile saline buffer. The serial dilutions of the samples at the concentrations of 25, 12.50, 6.25, 3.12, 1.56, 0.78, and 0.39 mg/mL were prepared in Mueller Hinton broth (CM0337, Oxoid). These dilutions were subsequently transferred to the wells of 96-well plates. A 20 µL of each bacterial inoculum was added to the wells, and the plates were incubated for 24 hours at 37 °C (Keyvan et al., 2022). After the incubation period, 20 µL of 2,3,5-triphenyl-tetrazolium chloride (1% TTC, Sigma-Aldrich) was added to the wells for each bacterial species to determine the MIC value. The plates were left for incubation at 37 °C for 3 hours. Red color shift in those wells showed the presence of metabolically active microorganisms (Karpinski, 2019). The minimum bactericidal concentration (MBC) value was obtained by subculturing the MIC dilutions. A 10 µL of the suspension was collected from each well and plated onto the Mueller Hinton agar (CM0337, Oxoid) plates. After the incubation for 24 hours, at 37 °C, the lowest extract concentration that inhibits bacterial growth was noted as the MBC value (Zielińska et al., 2021; Kahraman et al., 2022).

RESULTS

In the current study, the total phenolic content of the aqueous extract of the bee breed was determined using the Folin-Ciocalteu reagent. The TPC of bee bread was found to be 24.45 ± 3.75 µg of GAE/mg of dry extract. The scavenging activity of the extract has been determined by using a DPPH assay. The assay results demonstrated that the aqueous extract (1 mg/mL) of bee bread has shown to be 3.40 ± 2.99% scavenging activity. Ascorbic acid used as standard at the 50 µg/mL concentration had 89.76 ± 1.00% scavenging activity.

Table 1. MIC and MBC values of the aqueous extract of bee bread against tested bacteria.

	S. aureus	*E. coli*	*S.* Typhimurium	*B. cereus*
MIC (mg/mL)	6.25	6.25	6.25	12.50
MBC (mg/mL)	25.00	12.50	12.50	25.00

MIC: minimal inhibitory concentration, MBC: minimum bactericidal concentration

In the current study, the MIC values of bee bread extract against three bacteria were determined via the microdilution method. Subcultures of MIC dilutions were used to obtain MBC values. The MIC value against *B. cereus* was 12.50 mg/

mL. The MIC values against three other bacteria were determined at 6.25 mg/mL. The MBC values against *E. coli* and *S.* Typhimurium were 12.50 mg/mL. The MBC values against two other bacteria were determined at 25.00 mg/mL. The MIC and MBC values for the bee bread extract's antibacterial effects on *S. aureus*, *E. coli*, *S.* Typhimurium, and *B. cereus* are shown in Table 1.

DISCUSSION

Bee bread is a nutrient-dense substance that contains high levels of proteins, vitamins, and polyphenols, including phenolic acids. Phenolic compounds are considered to be a vital human dietary component and the largest contributors to the antioxidant potential of natural foods as well as other health benefits (Kumar and Goel, 2019). Several papers reported that the TPC in bee bread ranged from 2.1 to 25.4 mg of GAE/g (Ivanišová et al., 2015; Zuluaga et al., 2015; Suleiman et al., 2021). In the current study, the TPC of bee bread has been found as 24.45 ± 3.75 µg of GAE/mg of dry extract, corroborating previous studies.

The scavenging activity of the extract has been determined by using a DPPH assay. The assay results demonstrated that the aqueous extract (1 mg/mL) of bee bread has shown to be 3.40 ± 2.99% scavenging activity. Ascorbic acid used as standard at the 50 µg/mL concentration had 89.76 ± 1.00% scavenging activity. According to the findings of Dervişoğlu et al. (2022), the ethanolic extract from bee bread exhibited varying degrees of inhibition, ranging from 20.15 ± 0.68% to 93.18 ± 0.44%, depending on the concentration (ranging from 25 to 200 mg/mL). Akhir et al. (2017) reported that 70% ethanolic and hexane extract of bee bread exhibited percentage of inhibition 93.60 ± 0.03 and 83.81 ± 0.05, respectively. Another study showed that significant decreases in DPPH activity of bee bread from Malaysia were detected in bee bread water extract (7.62 ± 0.13%) relative to bee bread hot water extract (8.47 ± 0.01) and bee bread ethanolic extract (85.79 ± 0.40%) (Suleiman et al., 2021). The ethanolic extract of bee bread showed higher antioxidant properties due to higher phenolic and flavonoid contents compared to its aqueous extract (Othman et al., 2019). In the current study, the low antiradical activity of the aqueous extract may be due to the geographical characteristics of the region where bee bread is produced and the extraction method that changed the chemical composition of the extract.

In this study, MIC and MBC were used to determine the extract's antibacterial activities on *S. aureus, E. coli, S.* Typhimurium, and *B. cereus*. Pelka et al. (2021) observed an antibacterial effect on *S. aureus* at 2.5% (v/v) MIC concentration of bee bread. It has been reported that bee bread has a higher antibacterial effect on Gram-positive bacteria than pollen (Pelka et al., 2021). In a study conducted in Malaysia, the MIC_{50} values of bee bread were reported to be 1.914 µg/mL, 1.923 µg/mL, 1.813 µg/mL, and 1.617 µg/mL on *Klebsiella pneumonia, E. coli, Shigella,* and *S.* Typhi, respectively (Suleiman et al., 2021). In another study carried out in Ukraine, the antibacterial effect of bee bread with a MIC value of 6.40 µg/mL was determined on *E. coli* and *S. enterica* (Ivanišová et al., 2015). The application of different extraction methods and the diversity of plant flora may have been effective among the reasons for the varied results in MIC and MBC values in this study.

There are few studies about the water extract of bee bread. In a study conducted by Urcan et al. (2018), a concentration of 33% and 16.66% of bee bread water extract exhibited high inhibitory effects on *S. aureus*, whereas the partial inhibitory effect on *E. coli*, *S.* Enteritidis, *P. aeruginosa* and *B. cereus*. Sawicki et al. (2022) examined the antimicrobial effect of methanolic extract from bee bread and reported that the MIC values against to *S. aureus, L. monocytogenes, E. coli, E. faecalis* and *S.* Typhimurium ranging between 50% - 15% (v/v). Another study on ethanolic and hexane extract of stingless bee bread showed that MIC for *B. subtilis, S. aureus, E. coli* and *Salmonella* ranged from <6.67 to 33.33 µL/mL (Akhir et al., 2017).

CONCLUSION

Bee bread, which is made of pollen blended with honey and digestive enzymes from bees, is a treasured bee product that has been ignored. The results of this study showed that the bee bread from Ankara has antiradical activity and antibacterial effects against the foodborne pathogens.

DECLARATIONS

Ethics Approval

Ethics committee approval is not required since humans/animals were not used in our study

Conflict o Interest

Authors do not have any conflict of interests to disclose nor do they endorse the use of any product/technology/service over the other.

Consent for Publication

Not applicable

Competing Interest

The authors declare that they have no competing interests.

Author contribution

Idea, concept and design: NK, HAK, HT, EK

Data collection and analysis: NK, MSU, MMK, HAK, HT, EK

Drafting of the manuscript: NK, HAK, HT, EK

Critical review: NK, HAK, HT, EK

Data Availability

Not applicable.

Acknowledgments

Not applicable.

REFERENCES

1. Akhir, R. A. M., Bakar, M. F. A., & Sanusi, S. B. (2017). Antioxidant and antimicrobial activity of stingless bee bread and propolis extracts. In AIP conference proceedings, 1891(1), 020090. AIP Publishing LLC.

2. Bakour, M., Laaroussi, H., Ousaaid, D., El Ghouizi, A., Es-Safi, I., Mechchate, H., & Lyoussi, B. (2022). Bee bread as

a promising source of bioactive molecules and functional properties: an up-to-date review. Antibiotics, 11(2), 203. https://doi.org/10.3390/antibiotics11020203

3. Bayram, N. E., Gercek, Y. C., Çelik, S., Mayda, N., Kostić, A. Ž., Dramićanin, A. M., & Özkök, A. (2021). Phenolic and free amino acid profiles of bee bread and bee pollen with the same botanical origin–similarities and differences. Arabian Journal of Chemistry, 14(3), 103004. https://doi.org/10.1016/j.arabjc.2021.103004

4. Ćirić, J., Haneklaus, N., Rajić, S., Baltić, T., Lazić, I. B., & Đorđević, V. (2022). Chemical composition of bee bread (perga), a functional food: A review. Journal of Trace Elements and Minerals, 100038.

https://doi.org/10.1016/j.jtemin.2022.100038

5. Clinical and Laboratory Standards Institute-CLSI. (2016). Performance standards for antimicrobial susceptibility testing. Approved standard (M100S). (26th ed) Clin. Lab. Standard. Inst. Publ, Pennsylvania, USA.

6. Dervişoğlu, G., Çobanoğlu, D. N., Yelkovan, S., Karahan, D., Cakir, Y., & Koçyiğit, S. (2022). Comprehensive study on beebread: palynological analysis, chemical composition, antioxidant and cytotoxic activities. International Journal of Secondary Metabolite, 9(2), 166-177. https://doi.org/10.21448/ijsm.1066884

7. Didaras, N. A., Karatasou, K., Dimitriou, T. G., Amoutzias, G. D., & Mossialos, D. (2020). Antimicrobial activity of bee-collected pollen and beebread: State of the art and future perspectives. Antibiotics, 9(11), 811. https://doi.org/10.3390/antibiotics9110811

8. Ivanišová, E., Frančáková, H., Kačániová, M., Petrová, J., Hutková, J., Brovarskyi, V., Velychko, S., Adamchuk, L., Schubertová, Z., & Musilová, J. (2015). Bee bread-perspective source of bioactive compounds for future. Potravinarstvo, 9(1), 592-598. https://doi.org/10.5219/558

9. Kahraman, H. A., Tutun, H., Kaya, M. M., Usluer, M. S., Tutun, S., Yaman, C., Sevin, S., & Keyvan, E. (2022). Ethanolic extract of Turkish bee pollen and propolis: phenolic composition, antiradical, antiproliferative and antibacterial activities. Biotechnology & Biotechnological Equipment, 36(1), 44-55. https://doi.org/10.1080/13102818.2022.2045217

10. Kahraman, H. A., Tutun, H., Keyvan, E., & Balkan, B. M. (2022). Bioactive components, antibacterial and antiradical properties of home-made apple and grape vinegar. Ankara Üniversitesi Veteriner Fakültesi Dergisi, 69(2), 139-148. https://doi.org/10.33988/auvfd.865309

11. Kambur, M., & Kekeçoğlu, M. (2020). Is The Natural Honey Bee Biodiversity of Anatolia in the Process of Extinction?. Yüzüncü Yıl Üniversitesi Tarım Bilimleri Dergisi, 30(3), 593-600. https://doi.org/10.29133/yyutbd.708186

12. Karpiński, T. M. (2019). Efficacy of octenidine against Pseudomonas aeruginosa strains. European Journal of Biological Research, 9(3), 135-140. https://doi.org/10.5281/zenodo.3339499

13. Keyvan, E., Tutun, H., Kahraman, H. A., Şen, E., Demirtaş, A., Dönmez, S., & Akyüz, A. Ö. (2022). Determination of time dependent antibacterial activities of curcumin, carvacrol and styrax liquidus on Salmonella Enteritidis. Ankara Üniversitesi Veteriner Fakültesi Dergisi, 69(4), 355-360. https://doi.org/10.33988/auvfd.911244

14. Khalifa, S. A., Elashal, M., Kieliszek, M., Ghazala, N. E., Farag, M. A., Saeed, A., Xiao, J., Zou, X., Khatib, A., Göransson, U., & El-Seedi, H. R. (2020). Recent insights into chemical and pharmacological studies of bee bread. Trends in Food Science & Technology, 97, 300-316. https://doi.org/10.1016/j.tifs.2019.08.021

15. Kumar, N., & Goel, N. (2019). Phenolic acids: Natural versatile molecules with promising therapeutic applications. Biotechnology Reports, 24, e00370. https://doi.org/10.1016/j.btre.2019.e00370

16. Mohammad, S. M., Mahmud-Ab-Rashid, N. K., & Zawawi, N. (2020). Botanical origin and nutritional values of bee bread of stingless bee (Heterotrigona itama) from Malaysia. Journal of Food Quality, 2020, 1-12. https://doi.org/10.1155/2020/2845757

17. Othman, Z. A., Noordin, L., Ghazali, W. S. W., Omar, N., & Mohamed, M. (2019). Nutritional, phytochemical and antioxidant analysis of bee bread from different regions of Malaysia. Indian Journal of Pharmaceutical Sciences, 81(5), 955-960. https://doi.org/10.36468/pharmaceutical-sciences.590

18. Pełka, K., Otłowska, O., Worobo, R. W., & Szweda, P. (2021). Bee bread exhibits higher antimicrobial potential compared to bee pollen. Antibiotics, 10(2), 125. https://doi.org/10.3390/antibiotics10020125

19. Sawicki, T., Starowicz, M., Kłębukowska, L., & Hanus, P. (2022). The profile of polyphenolic compounds, contents of total phenolics and flavonoids, and antioxidant and antimicrobial properties of bee products. Molecules, 27(4), 1301. https://doi.org/10.3390/molecules27041301

20. Sobral, F., Calhelha, R. C., Barros, L., Dueñas, M., Tomás, A., Santos-Buelga, C., Vilas-Boas, M., & Ferreira, I. C. (2017). Flavonoid composition and antitumor activity of bee bread collected in northeast Portugal. Molecules, 22(2), 248. https://doi.org/10.3390/molecules22020248

21. Suleiman, J. B., Mohamed, M., Abu Bakar, A. B., Nna, V. U., Zakaria, Z., Othman, Z. A., & Aroyehun, A. B. (2021). Chemical profile, antioxidant properties and antimicrobial activities of Malaysian Heterotrigona itama bee bread. Molecules, 26(16), 4943. https://doi.org/10.3390/molecules26164943

22. Suna, B. (2019). Assessment of api tourism in Turkey by SWOT analysis. Uludağ Arıcılık Dergisi- Uludag Bee Journal, 19(1), 12-18. https://doi.org/10.31467/uluaricilik.568241

23. Tutun, H., Kaya, M. M., Usluer, M. S., & Kahraman, H. A. (2021). Bee pollen: Its antioxidant activity. Uludağ Arıcılık

Dergisi, 21(1), 119-131. https://doi.org/10.31467/uluaricilik.896045

24. Urcan, A., Crıste, A., Dezmırean, D., Bobış, O., Mărghıtaş, L., Mărgăoan, R., & Hrınca, A. (2018). Antimicrobial Activity of Bee Bread Extracts Against Different Bacterial Strains. Bulletin of the University of Agricultural Sciences & Veterinary Medicine Cluj-Napoca. Animal Science & Biotechnologies, 75(2). https://doi.org/10.15835/buasvmcn-asb:2018.0004

25. Zielińska, S., Dziągwa-Becker, M., Junka, A., Piątczak, E., Jezierska-Domaradzka, A., Brożyna, M., Paleczny, J., Sobiecka, A., Słupski, W., Mess, E., Kucharski, M., Çiçek, S. S., Zidorn, C., & Matkowski, A. (2021). Screening Papaveraceae as novel antibiofilm natural-based agents. Molecules, 26(16), 4778. https://doi.org/10.3390/molecules26164778

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Research Article

Decreased gene expression of RIPK1 and RIPK3, necroptosis players, in calves with sepsis

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ABSTRACT

As the world's population increases, so does the need for livestock-based nutrition. In addition, the livestock sector becomes more important as it contributes to the economy. However, sepsis has high morbidity and mortality rate in newborn calves and can cause severe economic losses. Therefore, new biomarkers to distinguish sepsis from other diseases are urgently needed in veterinary medicine. This study, for the first time, examined the gene expression levels of members of the necroptosis pathway, such as receptor-interacting serine/threonine protein kinase 1 (RIPK1), RIPK-3, and one of the NF-κB activating proteins, RIPK-2, in septic calves. We examined the mRNA levels of RIPK1, RIPK3, and RIPK2 using qPCR in 10 healthy Holstein calves and 20 Holstein calves with sepsis due to suffering from enteritis infection between 1-20 days of age. The hematologic parameters, including leukocytes, erythrocytes, hemoglobin, hematocrit, and platelets, were also evaluated in the calves included in this study. The results showed that calves with sepsis had prominently lower mRNA levels of RIPK1 and RIPK3 than those in healthy calves. Besides, RIPK2 mRNA expression was absent in healthy calves and calves sepsis. In veterinary medicine, decreased RIPK1 and RIPK3 mRNA levels might be biomarkers to diagnose sepsis in calves.

INTRODUCTION

The livestock sector provides animal protein to society and raw materials to the dairy, textile, leather, cosmetics, and pharmaceutical industries. Therefore, livestock is essential for all countries, as it contributes significantly to the economy. Sepsis following diarrhea can cause severe economic losses in calves (Fecteau et al., 1997; Guzelbektes et al., 2022). Sepsis is a complex overreaction described as the systemic hyperinflammatory immune response associated with a proven or suspected infection in animals and humans (Jarczak et al., 2021). In humans, it can cause multi-organ failure and lead to death in up to fifty percent of cases (Nedeva et al., 2019). Organ failure occurs after strong immune system activation by initiating the inflammatory response to eliminate pathogens by binding pathogen-associated molecular patterns (PAMPs) to their recognition receptors. Damage-associated molecular patterns (DAMPs) are then released by dying host cells to trigger the elevation of inflammatory cytokine levels that amplify cytokine storms. Organ failure and excessive organ infiltration of leukocytes are hallmarks of severe sepsis caused by cytokines, PAMPs, and DAMPs that can be released into the circulation from dying cells (Nedeva et al., 2019).

Necroptosis, a distinctive form of regulated non-apoptotic cell death, is triggered by death receptors such as tumor necrosis factor receptor 1 (TNFR1), TNFR2, and Fas. Receptor-interacting serine/threonine protein kinase 1 (RIPK1), RIPK3, and mixed lineage kinase domain-like protein (MLKL) which is a direct executor, are central to necroptosis. The translocation of MLKL to the cell membrane results in the rupture of the membrane and the release of intracellular contents and organelle swelling. In particular cell types and conditions, some PAMPs and DAMPs are associated with necroptosis induction (Kaczmarek et al., 2013). Studies revealed that the mortality of mice with TNF-induced systemic inflammatory response syndrome (SIRS) was driven by RIPK1-RIPK3-mediated necroptotic cell death (Duprez et al., 2011). In mice subjected to polymicrobial sepsis, RIPK3 promoted sepsis to induce acute kidney injury (Duprez et al., 2011). Moreover, necroptosis caused hepatic damage in piglets with lipopolysaccharide-induced sepsis (Xu et al., 2021). RIPK1 and RIPK3 activities have been linked to necroptosis. At the same time, RIPK2 plays essential roles in innate immunity and in the activation of the NF-kb, which has been implicated in the pathogenesis of organ injury and lethality during sepsis (Li et al., 2009; Bullock et al., 2015).

In human patients with sepsis, procalcitonin, and presepsin

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are currently used as biomarkers to detect sepsis, and new biomarkers have been investigated for precise diagnosis of sepsis. In addition, most acute phase proteins, including C-reactive protein, are used as biomarkers in animals with suspected sepsis. However, the amount of acute-phase proteins can also be elevated in other inflammatory diseases. In addition, sepsis and sepsis-related mortality rates in calves are poorly explored, but it is estimated that up to 30% of calves with neonatal diarrhea or illness are septic (Lofstedt et al., 1999; Fecteau et al., 2009). Thus, there is a need for new biomarker studies in terms of early diagnosis and prognostic follow-up of sepsis. Therefore, we evaluated the mRNA expression levels of RIPK1, RIPK3, and RIPK2 in calves with sepsis.

MATERIALS and METHODS

Animals

This study included10 healthy (control group) and 20 sepsis (experimental group) Holstein calves aged 1-20 days. In addition, routine clinical examinations of the diarrheal calves brought to the large cattle clinic were performed, and calves with sepsis, according to clinical and laboratory findings, were included in this study. The ethics committee approval required was obtained by Burdur Mehmet Akif Ersoy University Animal Experiments Local Ethics Committee on 16/12/2018. The presence of at least two or more recorded SIRS signs proven infection or infection with reduced or absent sucking reflex, lack of interest in the surroundings, inability to stand up without support, or lateral recumbency was defined as sepsis (Fecteau et al., 1997; Fecteau et al., 2009; Singer et al., 2009; Aygun and Yildiz, 2018).

Sample collection and analysis Blood samples for hemogram analyses from the experimental and control groups were taken from the *jugular vein*before treatment during routine blood tests. Tubes with ethylenediaminetetraacetic acid (K_3EDTA) were used for hemogram measurement, tubes with activator gel for serum and whole blood was used for RNA extraction and qPCR. A hemogram analysis was performed within 15-30 minutes. In addition, parameters such as leukocytes (WBC), granulocytes, erythrocytes (RBC), hematocrit (HCT), and platelets (PLT) from the venous blood sample with K_3EDTA were measured using the Abacus Junior Vet (Diatron MI Ltd. Hungary).

RNA extraction

Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used to extract the total RNAs from blood samples according to the manufacturer's instructions to identify RIPK1, RIPK2, and RIPK3 gene expression levels. RNAse-free water was used for the dissolution of RNAs. The integrity of RNAs was demonstrated on %1 agarose gel, and the concentration of these RNAs was analyzed using the BioTek spectrophotometer (Epoch, BioTek, Germany).

Real-time quantitative Polymerase Chain Reaction (qPCR)

The mRNA expression levels of RIPK1, RIPK2, and RIPK3 in samples of interest were quantified using Real-time quantitative PCR (qPCR). cDNA was then synthesized from RNA using the iScript Reverse Transcription kit (Biorad, Hercules, CA, USA) following the protocol provided by the manufacturer. qPCR analysis of RIPK1, RIPK2, and RIPK3 mRNA expressions in healthy and septic Holstein calf blood samples was performed using the SYBR Green PCR Master Mix on LightCycler 480 Instrument II (Roche, Basel, Switzerland). GAPDH was used to normalize RIPK1, RIPK2, and RIPK3 mRNA levels. Table 1 shows the sequences of primers used in the present study. The amplification parameters for qPCR were as 95°C for 10 min, followed by 50 cycles of 95°C for 10 sec, 65°C for 30 sec, and 72°C for 1 sec.

Statistical analysis

GraphPad Prism 6 for Windows® package program was used to analyze the obtained data statistically. During the statistical evaluation of the data, an unpaired t-test was used to determine the significant differences between the two groups in terms of a parameter. $p < 0.05$ values were considered statistically significant.

RESULTS

Clinical assesment and hemogram finding

Decreased sucking reflex, depression, lack of interest in consciousness, weakness in standing up, hypothermia or hyperthermia, prolongation of capillary filling time, abdominal respiration, hyperemia or pallor of mucous membranes were observed in calves with sepsis. The hematological and clinical parameters, including body temperature, respiration, and pulse rate of the calves of the experimental and control groups and are presented in Table 2. In the hemogram analysis, leukocyte (WBC) ($p < 0.01$) and granulocyte ($p \leq 0.01$) counts were significantly increased in calves with sepsis compared to healthy calves. However, RBC, HCT and PLT levels were not found to be statistically significant. In addition ieukopenia was detected

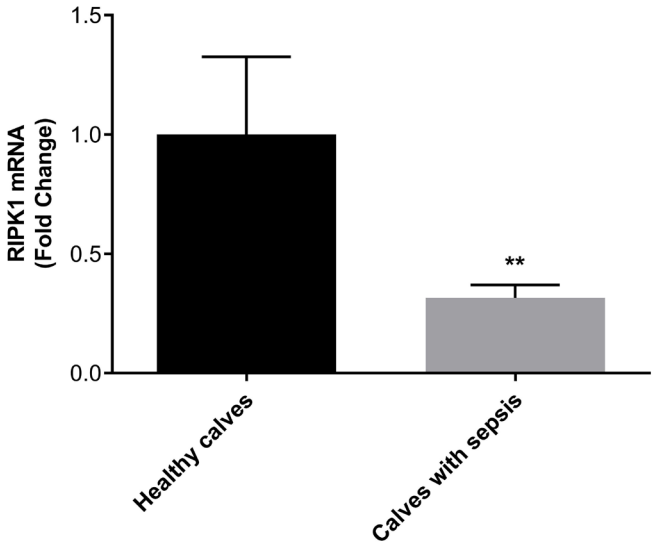

Figure 1. Decreased RIPK1 mRNA expression in calves with sepsis. The mRNA expression levels RIPK1 were examined in blood samples of health calves (n=10), and in blood samples of age-matched calves with sepsis (n=20) using qPCR. GAPDH was used to normalize RIPK1 mRNA levels. Values indicate mean ± SEM. Student unpaired t-test **$p < 0.01$ ($p=0.0054$).

Table 1. Sequences and annealing temperature (ºC) of primers used in this study

Gene Name	Primer Sequence (5'-3')	Annealing temperature (ºC)
RIPK1-F	5' ATTCCATTCACCTCCTTGCC 3'	57
RIPK1-R	5' GAACTCATTCCACCAATCTCCA 3'	58
RIPK2-F	5' CAGTGAATCACAGTCGGAACAG 3'	60
RIPK2-R	5' AAGCACAAGTATTTCTGGGTAAGG 3'	59
RIPK3-F	5' CCGAATAAACACCAAGAAAGCC 3'	58
RIPK3-R	5' TCAGAAAGGAACTCCTTCACCA 3'	58
GAPDH-F	5' GGTCACCAGGGCTGCTTTTA 3'	59
GAPDH-R	5' AGGATCTCGCTCCTGGAAGA 3'	59

Table 2. Hemogram and clinical findings of sepsis and control groups (Mean±SEM)

Parameters	Control (n:10)	Sepsis (n:20)	p value
WBC$^{(10^9/l)}$	8.18±0.49	21.2±3.79	0.003
GRA$^{(10^9/L)}$	3.65±0.49	13.5±3.34	0.008
RBC$^{(10^12/l)}$	8.74±0.73	10.2±2.38	0.552
HCT (%)	27.9±2.21	29.3±1.84	0.626
PLT$^{(10^9/l)}$	572±121	670±76.2	0.504
Temperature (°C)	38.9±0.09	37.3±0.29	0.00
Breath (min)	36.3±0.73	45.6±4.08	0.04
Pulse (min)	118±2.72	105±6.20	0.06
Gender	6 male, 4 female	7 male, 13 female	

WBC = white blood cell, GRA = granulocytes. RBC = red blood cells, HCT = haematocrit, PLT = platelets.

in 2 calves, and leukocytosis was detected in 18 calves with sepsis.

Results of the mRNA expression levels of RIPK1, RIPK2, and RIPK3RIPK1 mRNA expression levels were statistically decreased approximately threefold in calves with sepsis compared to healthy calves based on qPCR results (Figure 1).Similarly, RIPK3 mRNA levels were lower in calves with sepsis compared to their controls (Figure 2).

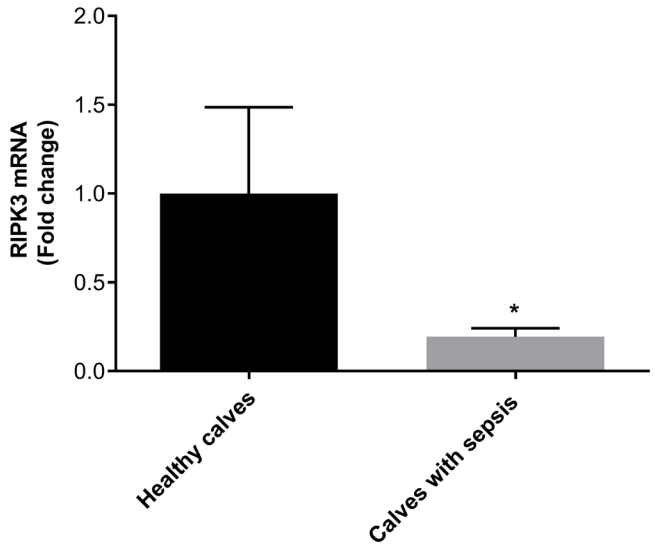

Figure 2. Lower RIPK3 mRNA expression in calves with sepsis. RIPK3 expression was evaluated by qPCR in blood samples of health calves (n=10), and in blood samples of age-matched calves with sepsis (n=20). Values indicate mean ± SEM. Student unpaired t-test **p < 0.05 (p=0.0267).

However, RIPK2 expression could not be determined in both the healthy calves and the calves with sepsis, probably due to its low expression (graph not provided).

DISCUSSION

Neonatal sepsis remains one of the most significant health problems in cattle breeding because of its high morbidity and mortality rate. Despite the development of sepsis treatment options, new biomarkers are needed for early diagnosis and prognostic follow-up of sepsis. In this study, the mRNA expression levels of RIPK1 and RIPK3, crucial players in the necroptosis cell death pathway, were investigated for the first time. We found that the gene expression levels of RIPK1 and RIPK3 were significantly decreased in the calves with sepsis compared to those in healthy calves. Moreover, pro-inflammatory inducer RIPK2 expression was not detected in calves by qPCR.

Sepsis has a complex and multifactorial pathophysiology. In general, the pro-inflammatory process is stimulated by the pathogen, and it accompanies the elimination of the pathogen, but the host activates the anti-inflammatory process to repair the tissues. The imbalance of these mechanisms can cause excessive tissue damage (pro-inflammatory) or immunosuppression in the body and predispose it to secondary infections

(anti-inflammatory). The organ's response to these events depends on the features of the defense of the host (morbidity and immunosuppression) and the pathogen (virulence and amount of organism) (Angus and Van der Poll, 2013). Numerous defense mechanisms are emerging for pathogen control and elimination, including inflammatory cytokine, interferon, and chemokine production, adaptive immune response, and activation of cell death pathways, including necroptosis (Fecteau et al., 1997; Guzelbektes al., 2022). Necroptosis is a genetically regulated necrotic cell death pathway that is thought to kill pathogen-infected cells and/or damaged cells in inflammatory pathologies. Necroptosis is mediated by TNFR and Fas, or pathogen recognition receptors, including tTLRs and Z-DNA binding protein 1 (Qu et al., 2022). RIPK1, RIPK3 and MLKL have been identified as critical molecular components of necroptosis (He et al., 2000; Cho et al., 2009; Holler et al., 2009; Zhang et al., 2009). The binding of TNF to TNFR leads to the recruitment and phosphorylation of RIPK1, the adaptor protein TRADD (death domain-associated TNFRSF1A). RIPK1 then activates RIPK3, which in turn phosphorylates MLKL kinase. After phosphorylation, MLKL undergoes significant conformational changes that allow its insertion into the cell membrane and induce membrane permeability by creating pores.RIPK3 has been identified as the essential molecule for TNF-induced necroptosis (Upton et al., 2010; Moujalled et al., 2013), while RIPK1 has an essential role in mediating both TNF-dependent nuclear factor κB (NFκB) activation and apoptosis (Ofengeim and Yuan, 2013). In the present study, RIPK1 and RIPK3 mRNA levels were lower in the calves with sepsis (experimental group) than in the healthy calves. Duprez et al.'s (2011) study emphasized that inhibition of RIPK1 and RIPK3 is essential in preventing SIRS caused by TNF. Studies showed that necroptosis increases in direct proportion to the severity of sepsis when severe sepsis and septic shock were compared with sepsis in humans (Duprez et al., 2011; Wang et al., 2012). Evidence supporting our study was the relationship between inflammation and necroptosis in a mouse model of TNF-induced SIRS (Kaiser et al., 2013). RIPK3 deficiency has been reported to protect against lethal SIRS and reduce circulating DAMPs (Kaiser et al., 2013). It is thought that the calves with sepsis taking place in our study may be in the initial stage of sepsis, and the RIPK3 level may decrease temporarily, but multi-organ dysfunction findings will occur due to the development of necroptosis and apoptosis in the later stages. A recent study revealed that RIPK3 cooperates with the lethal gesdarmin D (GSDMD), which is involved in pyroptosis, to increase tissue damage in polymicrobial sepsis (Chen et al., 2020). Briefly, this study reported that both necroptosis and pyroptosis are associated with caecal ligation and perforation (CLP)-induced sepsis and multi-organ dysfunction, and the use of agents that inhibit the activity of these molecules may protect against septic shock and multi-organ damage (Chen et al., 2020).

Han et al. (2019) showed that necroptosis is involved in the pathogenesis of acute-on-chronic hepatitis B liver failure (ACHBLF). For that, they measured the mRNA levels of RIPK3 using qPCR and the protein levels of MLKL by ELISA in the peripheral blood of healthy controls and patients with ACHBLF and chronic hepatitis B. They found that RIPK3 mRNA levels were significantly higher in patients with ACHBLF than those with chronic hepatitis B or healthy controls, positively correlated with serum MLKL. Moreover, the mRNA expression of MLKL, RIPK3, and Beclin-1 was assessed in 45 primary immune thrombocytopenia (ITP) patients' peripheral blood and 20 healthy controls' peripheral blood. The parameters of clinic and laboratory and patients' response to steroid therapy were evaluated with mRNA expression of MLKL, RIPK3, and Beclin-1. It was found that there is a crosstalk between increased mRNA expression of RIPK3 and MLKL and autophagy-related protein Beclin-1 in primary immune thrombocytopenia (Kamal et al., 2022). Moreover, the production of RIPK3 mRNA and protein expression showed correlation with the presence of necroptosis signaling in melanoma cell lines (Geserick et al., 2015). All these studies indicate the correlation between mRNA and protein levels of RIPK3.

RIPK2 plays an essential role in forming the immune response by intracellular nucleotide binding and oligomerization domain (NOD)-mediated NF-kB activation and cytokine production (Nachbur et al., 2015; He et al., 2017). NOD receptors recognize antigens containing bacterial peptidoglycans and initiate immune responses by activating NF-κB and MAP kinases, triggering the production of pro-inflammatory cytokines (Jun et al., 2013). No study has been found investigating the potential role of RIPK2 in the occurrence of bacterial sepsis. In our study, the mRNA expression level of RIPK2, which has a role in forming the immune response against bacterial infection, could not be determined in most healthy and septic calves. This is thought to be due to the late arrival of RIPK2 in the cycle and the differences between individuals.

CONCLUSION

In summary, RIPK1 and RIPK3 may contribute to the early diagnosis and understanding of the therapeutic effect of neonatal sepsis in calves. The combined detection of RIPK3 with proven biomarkers such as c-reactive protein and procalcitonin may be more effective than individual ones in diagnosing neonatal sepsis. Moreover, studies are required to compare the changes in RIPK1 and RIPK3 expressions in the treatment processes of patient groups (sepsis, severe sepsis, and septic shock) with healthy calves. In addition, investigating the role of RIPK1 and RIPK3 in the treatment process in calves will contribute to understanding their potential to be used as mortality and prognostic indicators and the mechanisms that contribute to increasing treatment success. This study's results suggest that lower RIPK1 and RIPK3 mRNA levels may be associated with the beginning of sepsis. Furthermore, RIPK2 may not contribute to the induction of innate immunity in calves with sepsis.

DECLARATIONS

Ethics Approval

The present study was approved by the Animal Experiments Local Ethics Committee of Burdur Mehmet Akif Ersoy University (Protocol 16-12-2018).

Conflict o Interest

The authors have no conflicts of interest.

Consent for Publication

Not applicable.

Author contribution

Idea, concept and design: AD, DAY

Data collection and analysis: AD, YB, DAY

Drafting of the manuscript: AD, YB, DAY

Critical review: AD, YB, DAY

Data Availability

Data were available on request from correspondence author.

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REFERENCES

1. Angus, D., C., & Vander, P., T. (2013). Severe sepsis and septic shock. The New England Journal of Medicine, 369, 840-51.

2. Aygun, O., & Yildiz, R. (2018). Evaluation of thrombomodulin and pentraxin-3 as diagnostic biomarkers in calves with sepsis. Veterinarni Medicina, 63, 313–20.

3. Bullock, A., N., & Degterev, A. (2015). Targeting RIPK1,2,3 to combat inflammation. Oncotarget, 6(33), 34057-8. https://doi.org/10.18632/oncotarget.6106

4. Chen, H., Li, Y., Wu, J., Li, G., Tao, X., Lai, K., Yuan, Y., Zhang, X., Zou, Z., & Xu, Y. (2020). RIPK3 collaborates with GSDMD to drive tissue injury in lethal polymicrobial sepsis. Cell Death Differentiation, 27(9), 2568-2585. https://doi.org/10.1038/s41418-020-0524-1

5. Cho, Y., Challa, S., Moquin, D., Genga, R., Ray, T. D., Guildford, M., & Chan, F. K. (2009). Phosphorylation-driven assembly of the RIPK1-RIPK3 complex regulates programmed necrosis and virus-induced inflammation, Cell, 137, 1112–23.

6. Duprez, L., Takahashi, N., Van , H., F., Vandendriessche, B., Goossens, V., Vanden, B., T., Declercq, W., Libert, C., Cauwels, A., & Vandenabeele, P. (2011). RIP kinase-dependent necrosis drives lethal systemic inflammatory response syndrome. Immunity, 35(6), 908-18. https://doi.org/10.1016/j.immuni.2011.09.020

7. Fecteau, G., Paré, J., Van Metre, D. C., Smith, B. P., Holmberg, C. A., Guterbock, W., & Jang, S. (1997). Use of a clinical sepsis score for predicting bacteremia in neonatal dairy calves on a calf rearing farm. The Canadian Veterinary Journal, 38(2), 101-104.

8. Fecteau, G., Smith, B., P., & George, L., W. (2009). Septicemia and meningitis in the newborn calf. Veterinary Clinics of North America: Food Animal Practice, 25(1), 195-208.

9. Geserick, P., Wang, J., Schilling, R., Horn, S., Harris, P., A., Bertin, J., Gough, P. J., Feoktistova, M., & Leverkus, M. (2015). Absence of RIPK3 predicts necroptosis resistance in malignant melanoma. Cell Death & Disease 6(9), e1884. https://doi.org/10.1038/cddis.2015.240

10. Guzelbektes, H., Sen I., Aydogdu, U., Er, C., & Coşkun, A. (2022). Investigation of cytokine levels in calves with sepsis. Journal of the Hellenic Veterinary Medical Society, 73(2), 4113–4118. https://doi.org/10.12681/jhvms.26655

11. Han, L., Teng, Y., Fan, Y., Gao, S., Li, F., & Wang, K. (2019). Receptor-Interacting Protein Kinase 3 (RIPK3) mRNA Levels Are Elevated in Blood Mononuclear Cells of Patients with Poor Prognosis of Acute-on-Chronic Hepatitis B Liver Failure. Tohoku Journal of Experimental Medicine, 247(4), 237-245. https://doi.org/10.1620/tjem.247.237

12. He, S., Wang, L., Miao, L., Wang, T., Du, F., Zhao, L., & Wang, X. (2009). Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-α. Cell, 137, 1100–11.

13. He, X., Da, R., S., & Nelson, J. (2017). Identification of Potent and Selective RIPK2 Inhibitors for the Treatment of Inflammatory Diseases. ACS Med Chem Lett 8(10), 1048–1053.

14. Holler, N., Zaru, R., Micheau, O., Thome, M., Attinger, A., Valitutti, S., Bodmer, J. L., Schneider, P., Seed, B., & Tschopp, J. (2000). Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nature Immunology, 1, 489–495.

15. Jarczak, D., Kluge, S., & Nierhaus, A. (2021). Sepsis-Pathophysiology and Therapeutic Concepts. Frontiers Medicine (Lausanne), 8, 628302. https://doi.org/10.3389/fmed.2021.628302

16. Jun, J., C., Cominelli, F., & Abbott, D., W. (2013). RIP2 activity in inflammatory disease and implications for novel therapeutics. Journal of Leukocyte Biology, 94(5), 927–932.

17. Kaczmarek, A., Vandenabeele, P., & Krysko, D., V. (2013). Necroptosis: the release of damage-associated molecular patterns and its physiological relevance. Immunity, 38(2), 209-23. https://doi.org/10.1016/j.immuni.2013.02.003

18. Kamal, A., M., Nabih, N., A., Rakha, N., M., & Sanad, E., F., (2023). Upregulation of necroptosis markers RIPK3/MLKL and their crosstalk with autophagy-related protein Beclin-1 in primary immune thrombocytopenia. Clinical and Experimental Medicine, 23(2), 447-456. https://doi.org/10.1007/s10238-022-00839-8

19. Kaiser, W., J., Sridharan, H., Huang, C., Mandal, P., Upton, J., W., Gough, P., J., Sehon, C., A., Marquis, R., W., Bertin, J., & Mocarski, E., S., J. (2013). TRIF, RIP3 ve MLKL yoluyla otoyol benzeri reseptör 3 aracılı nekroz. Journal of Biological Chemistry, 288(43), 31.268-79.

20. Li, X., Su, J., Cui, X., Li, Y., Barochia, A., & Eichacker, P., Q. (2009). Can we predict the effects of NF-kappaB inhibition in sepsis? Studies with parthenolide and ethyl pyruvate. Expert Opinion on Investigational Drugs, 18(8), 1047-60. https://doi.org/10.1517/13543780903018880

21. Lofstedt, J., Dohoo, I., R., & Duizer, G. (1999). Model to predict septicemia in diarrheic calves. Journal of Veterinary Internal Medicine, 13(2), 81-88. https://doi.org/10.1892/0891-6640(1999)013<0081: Mtpsid>2.3.Co;2

22. Moujalled, D., M., Cook, W., D., Okamoto, T., Murphy, J., Lawlor, K., E., Vince, J., E., & Vaux, D., L. (2013). TNF can activate RIPK3 and cause programmed necrosis in the absence of RIPK1. Cell Death Discovery, 4(1), e465. https://doi.org/10.1038/cddis.2012.201

23. Nachbur, U., Stafford, C., & Bankovacki, A. (2015). A RIPK2 inhibitor delays NOD signalling events yet prevents inflammatory cytokine production. Nature Communications, 6, 6442. https://doi.org/10.1038/ncomms7442

24. Nedeva, C., Menassa, J., & Puthalakath, H. (2019). Sepsis: Inflammation Is a Necessary Evil. Frontiers in Cell and Developmental Biology, 7, 108. https://doi.org/10.3389/fcell.2019.00108

25. Ofengeim, D., & Yuan, J. (2013). Regulation of RIPK1 kinase signalling at the crossroads of inflammation and cell death. Nature Reviews Molecular Cell Biology, 14, 727–36.

26. Qu, M., Wang, Y., Qiu, Z., Zhu, S., Guo, K., Chen, W., Miao, C., & Zhang, H. (2022). Necroptosis, Pyroptosis, Ferroptosis in Sepsis and Treatment. Shock, 57(6), 161-171. https://doi.org/10.1097/SHK.0000000000001936

27. Singer, M., Deutschman, C., S., & Seymour, C., W. (2016). The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). JAMA 315(8), 801–810.

28. Upton, J. W., Kaiser W. J., & Mocarski E. S. (2010). Virus inhibition of RIPK3-dependent necrosis. Cell Host Microbe, 7, 302–313.

29. Wang, Z., Jiang, H., Chen, S., Du, F., & Wang, X. (2012). The mitochondrial phosphatase PGAM5 functions at the convergence point of multiple necrotic death pathways. Cell, 148, 228–243.

30. Xu, Q., Guo, J., Li, X., Wang, Y., Wang, D., Xiao, K., Zhu, H., Wang, X., Hu, CA., Zhang, G., & Liu, Y. (2021). Necroptosis Underlies Hepatic Damage in a Piglet Model of Lipopolysaccharide-Induced Sepsis. Front Immunol, 12, 633830. https://doi.org/10.3389/fimmu.2021.633830

31. Zhang, D. W., Shao J., Lin J., Zhang N., Lu B. J., Lin S.C., Dong M. Q., & Han J. (2009). RIPK3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science, 325(5938), 332-6. https://doi.org/10.1126/science.1172308

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Research Article

Intense exercise stress may trigger *Corynebacterium kutscheri* infection in Sprague-Dawley rats

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ABSTRACT

Corynebacterium kutscheri infection in rodents is usually related to stressful conditions. In this study, *C. kutscheri* infection has been reported that occurred as a complication in rats subjected to intense swimming stress during an experimental study. Weight loss, indifference to the environment, fluffy feathers, and hunched posture were observed in affected rats. The lungs contained numerous, randomly distributed, variably sized, slightly raised, cream-colored caseopurulent foci. There were occasional weak adhesions between the lung lobes and the adjacent costal pleura. Multifocal to coalescing necro-suppurative pneumonia with intralesional scattered large colonies of bacteria was observed histopathologically. Adjacent in the pulmonary parenchyma, interalveolar hypercellularity, type II pneumocyte hyperplasia, fibrinonecrotic vasculitis, and pleuritis were observed. Brown-Breen staining revealed gram-positive cocobacilli in the lesion areas. Furthermore, cardiac lesions in which the atria were more severely affected than the ventricles were identified. This lesion was characterized by thickening of the epicardium with intense infiltrates of macrophages admixed with scattered neutrophils. In severely affected rats, this lesion was also involved to the underlying myocardium. Bacterial culture yielded positive growth for *C. kutsheri* from the lesioned organ. Polymerase chain reaction was used to confirm the presence of genetic material for *C. kutscheri*. As a result, it was revealed that Sprague-Dawley rats were infected with *C. kutscheri* due to intense exercise stress. Periodic controls of *C. kutscheri* have been suggested in units where experimental animals are raised, both because of its negative effects on the results of the studies to be conducted and because of its zoonotic nature.

INTRODUCTION

The disease caused by *Corynebacterium kutscheri*, also known as pseudotuberculosis, was first described by Kutscheri in 1984 in mice (Lemaistre & Tompsett, 1952). Later research revealed that the disease also affects rats, hamsters, and guinea pigs (Otto et al., 2015). *C. kutscheri* is a diphtheroid gram-positive bacillus. It can be observed in different morphologies during growth for this reason also called "Chinese letter" (Won et al., 2007). Natural infection with *C. kutscheri* is generally subclinical (Amao et al., 1995). Early studies showed that this organism was commonly harbored latently in colonies of rodents. Bacteria are commonly found in the oral cavity, cecum, and oropharyngeal, cervical, and maxillary lymph nodes (Amano et al., 1991; Amao et al., 2002; Amao et al., 2008). Although good laboratory practices reduce the prevalence of the infection, it can occasionally be encountered with fetal epizootics (Barthold, 2012). Concurrent stressors are frequently associated with disease outbreaks. Transportation, overcrowding, malnutrition, immunosuppressive drugs, radiation, and experimental applications are among these factors (Amao et al., 2008). Latent infections can become active in the presence of these factors. The active infection that results most likely spreads hematogenously to various tissues and organs, causing necrosuppurative lesions (Giddens et al., 1968). The location of the lesions varies depending on the animal species. The lungs are the most severely affected organ in rats. In mice, the liver is the primary active site of infection (Barthold, 2012). Clinical findings differ depending on the affected organ, but they are not specific (Otto et al., 2015). It is necessary to remove any latently infected animals discovered in experimental animal facilities through periodic analysis to prevent the spread of infection. The definitive diagnosis of the disease is made according to the presence of characteristic histolopathologic lesions, bacteriological culture, and molecular analysis results (Fox et al., 1987; Jeong et al., 2013).So far, no infections with *C. kutscheri* have been reported in experimental production facilities in Turkey. Moreover, no clinical, pathological, or microbiological studies have been conducted to investigate the presence of *C. kutscheri* in experimental animals. This could be due to a lack of research on the subject rather than a lack of latent *C. kutscheri* infection in rodent colonies. The lack of scientific research on this subject may have an adverse effect on the outcomes of animal experiments (Saltzgaber-Muller & Stone, 1986). Aside from these, *C. Kutscheri*'s zoonotic nature poses significant potential risks to the occupational health and safety of researchers and assisting staff working in laboratory animal breeding units (Himsworth et al., 2014; Holmes & Korman, 2007). The clinical, gross, histopathological, bacteriological, and molecular findings of *C. kutscheri*-associated disease in a colony of Sprague-Dawley rats in a certified experimental animal breeding unit are presented in this study. In this context, we aimed to draw attention to the *C. kutscheri* disease among researchers who will be working with experimental animals and their personnel in the experimental animal breeding unit.

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MATERIAL and METHODS

Animals

The study's animal material included Sprague-Dawley rats that died or were euthanized as a result of a *C. kutscheri* infection that occurred as a complication during an experimental study. During the experiment, the rats were housed in standard temperature (22 ± 2°C), relative humidity (50%–70%), and lighting conditions (12/12-h light/dark). Ad libitum access to standard rat feed and tap water was provided. Thirty-two 10-week-old Sprague-Dawley rats were obtained from a certified commercial experimental animal breeding facility for the experiment. The rats were randomly divided into two groups of 16 rats each: forced swim groups and control non-swimming groups. The rats in the forced swim group had a weight equal to 5% of their body weight attached to their tails and were subjected to 1 h of swimming in 32°C pools 5 days a week (Claudio et al., 2013). The rats were dried after flotation before being placed in their cages. The rats in the control group received no treatment.

In the forced swim group, 1 rat (no. 1) died on the 10th day of the experiment and 2 rats (nos. 2–3) died on the 11th day of the experiment. The study was halted because the systemic necropsy findings of the dead rats indicated that the experiment's results would be harmed. All of the rats were examined, clinical symptoms were recorded, and they were euthanized humanely via cervical dislocation under general anesthesia induced with xylazine and ketamine.

Pathologic examination

Systemic necropsies were performed on dead and euthanized rats, and gross and subgross lesions were recorded. Identified macroscopic lesions were photographed (#c5050, Olympus), and tissue samples were fixed in 10% buffered formalin for 48 h and trimmed according to the proposed method of Ruehl-Fehlert et al. (2003) before loading into tissue follow-up cassettes. Tissue samples were dehydrated by passing them through a series of increasing alcohol concentrations, then cleared with xylene, and embedded in paraffin. With a microtome, serial sections of 4–5 µm thickness were cut from paraffin blocks and stained with hematoxylin–eosin (HE). Brown-Breen staining was used to highlight the agents in selected tissue sections. The tissue slides were examined with a light microscope (#BX51, Olympus), and photomicrographs were taken with a camera (#SC180, Olympus).

Bacteriological examination

Under aseptic conditions, samples from the lung, submandibular lymph node, spleen, liver, and preputial glands of all rats were inoculated on Columbia Agar plates with 5% sheep blood (#110025, Liofilchem®) for bacteriological examination. In both aerobic and microaerophilic environments, the media were incubated at 37°C for 24–48 h. Bacterial microscopic properties were determined by staining isolated bacterial colonies with the gram staining method. The biochemical properties of the cultured bacteria were characterized with VITEK 2® (bioMérieux).

Polymerase chain reaction

Polymerase chain reaction (PCR) was used to confirm the isolated *C. kutscheri* strains' molecular identities. Genomic DNA was isolated with the help of a High Pure PCR Template Preparation Kit (#11796828001, Roche) in line with the manufacturer's recommendations. Primers specific to C. kutscheri reported by Jeong et al. (2013) as (F: 5'-CGTGATGGCCATCTTTGGTT-3', R: 3'-AATCGTATTAGCAAAGGTATGC-5') were used. DNA was amplified with an Xpert Fast Hotstart Mastermix (2×) with dye kit (#GE45.0001, GRiSP) in a Techne TC-412 thermal cycler (Keison Products) using the following cycling conditions: 95°C hold 3 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 30 s, extension at 72°C for 15 s, and final elongation at 72°C for 3 min. The PCR products were loaded onto a 2% agarose gel and run at 100 V for 1 h before being visualized.

RESULTS

Clinical signs

The forced swimming rats showed rapid weight loss (12/16, 75%), indifference to the environment (12/16, 75%), fluffy hairs (12/16, 75%), and hunched posture (12/16, 75%) 1 week after the experiment began. In addition to these symptoms, periocular encrusted exudate (7/16, 43.75%), wheezing (7/16, 43.75%), and gasping (7/16, 43.75%) appeared later. On 10th (n=1) and 11th (n=2) days of the experiment, the rats were seen motionless on the floor with subnormal temperature and superficial respiration and subsequent death by passing into an agony state.

Gross finding

The control rats had no macroscopic lesions. The lungs of 12 of the rats in the forced swim group were heavier, edematous, and poorly collapsed. Suppurative foci of 1- to 6-mm size, cream-colored, and raised from the surface were found in the lungs of seven of these rats (Figure 1A). Some lesions were seen merged together, encompassing the entire affected lobe of the lung. The location and extent of lesions in the lobes of the lungs has been shown in Table 1.

There was no specific pattern of lesions in the lung lobes, and lesions were randomly distributed. Multiple caseonecrotic foci of variable sizes were seen on the cross-sectional surface of the lesion areas. In one case (no. 4), pus could be seen oozing from the lesion's cross-sectional surface, which was surrounded by a thick fibrous capsule (Figure 1B). Serous-to-fibrinopurulent foamy fluid was present in the tracheal lumen in five cases. The pleura had a ground-glass appearance in six cases (nos. 1–3, 5–7), and adhesions formed between the lung lobes and the pericardium and thorax in three cases (nos. 5–7). The pleural appearance was granular in the areas of adhesion. The apex of the heart was rounded in six rats, the myocardium was white mottled, particularly in the atria, and both ventricle lumens had coagulated blood. The pericardium had a ground-glass appearance in all six of these cases, and there were adhesions between the pericardial leaves. The liver was swollen and cyanotic in six rats, with blood oozing from the cut surface.

Table 1. Location and degree of lesions in the lobes of the lungs

No.	Lung				
	Left lobe	Right lobe			
		Cranial	Middle	Caudal	Accessory
1	+++	+++	+++	+++	+++
2	+++	-	-	-	-
3	+++	+++	+++	+++	+++
4	-	-	-	+++	-
5	-	+++	++	+++	-
6	+	-	-	+	+++
7	-	+++	++	+++	-

Note. According to the degree of involvement of lung lobes:–, no lesion; +, affected <25%; ++, affected 25%–50%; +++, affected >50%.

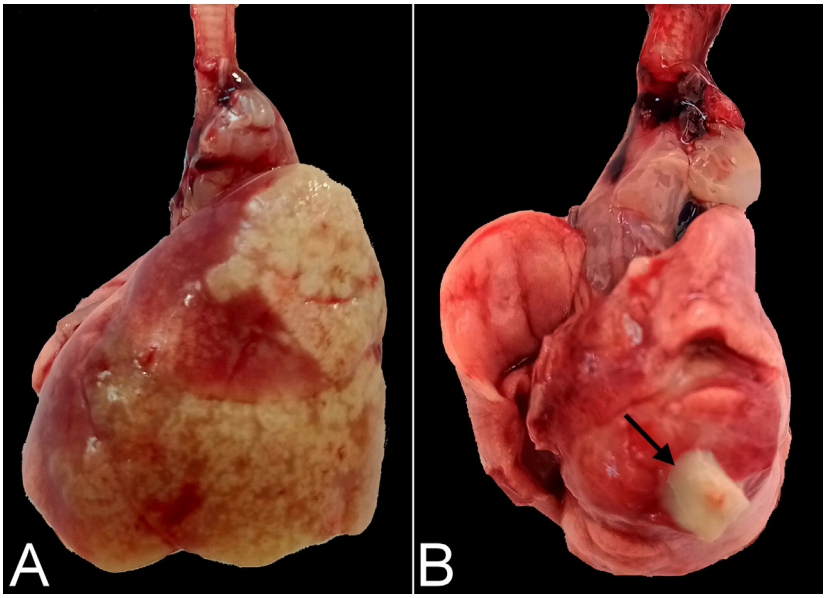

Figure 1. Corynebacterium kutsheri-associated gross lesions. A) Randomly distributed, varying sizes, slightly protruding from the surface, grayish-white caseo-purulent foci in the lungs; B) Single, large, raised abscess in the lung. Grayish-yellow pus oozing from the cross section of the lesion.

Histopathological finding

The lungs and hearts had the most visible microscopic lesions. In general, lung lesions were seen to begin with neutrophil infiltration to the interalveolar septum (12/16, 75%). At this stage, the alveolar lumens were empty (Figure2A).Hypercellularity in the interalveolar septum was occasionally accompanied by alveolar epithelization. As the lesions progressed, the alveolar lumens were seen to fill with inflammatory exudate containing shed epithelial cells, neutrophils, and macrophages (Figure 2B). In the lungs of seven rats, multifocal randomly distributed coagulative to caseous necrotic foci with abundant viable and degenerate neutrophils were observed (Figure 2C). Scattered colonies of basophilic bacteria were observed in the necrotic areas. Using Brown-Breen staining, these bacterial colonies in the necrotic areas were identified as gram-positive coccobacilli. Around neutrophil collections, macrophages, epithelioid histiocytes, and sporadic multinucleated giant cells were found on occasion (Figure 2D). Merging neighboring lesions resulted in larger lesions in severely affected lung lobes. In the immediate vicinity of the caseonecrotic foci, neutrophils with karyorrhectic nuclei formed a demarcation zone. (Figure 2E).

In one rat (no. 4), the lesions were surrounded by mature connective tissue from the outside. Bronchial, bronchiole, and tracheal epithelium was exfoliated in cases where the lung lobe was diffusely affected, and fibrinopurulent exudate covered the ulcerated mucosa. The vessels near the pyogranulomatous lesions had fibrinonecrotic vasculitis and occasional thrombosis (Figure 2F-G).

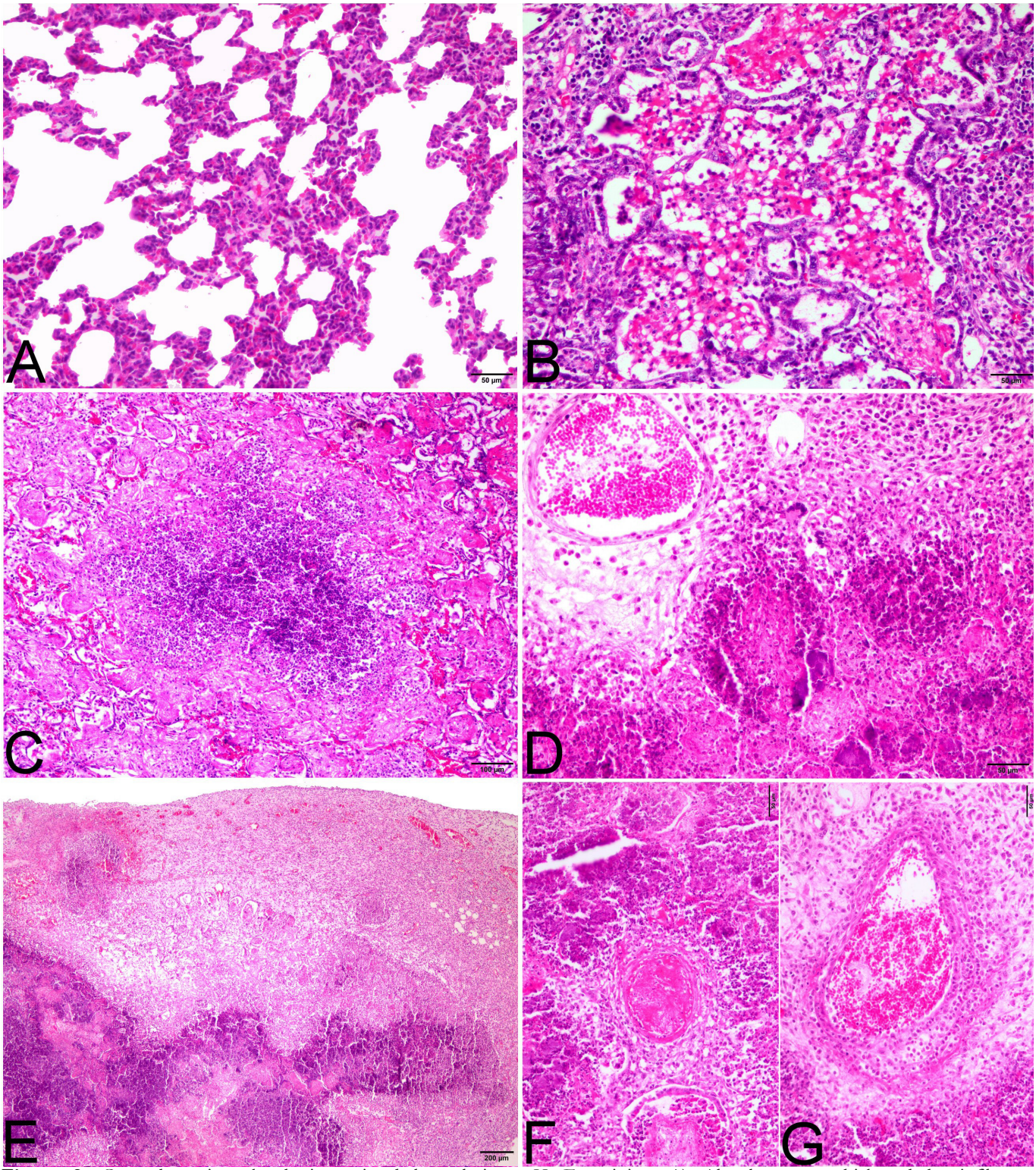

Figure 2. Corynebacterium kutsheri-associated lung lesions. H&E staining. A) Alveolar septa thickened by infiltrating neutrophils; B) Alveolar surfaces covered by cubic shaped pneumocytes (type II pneumocytes). Alveolar lumina contain homogenous edema fluid mixed with neutrophils; C) Note dark basophilic infiltrates of viable and degenerated neutrophils in a background of proteinaceous edema fluid; D) Accumulation of viable and degenerated neutrophils with intralesional large colonies of bacteria; E) Large coalesing areas of necrosis, infiltrated and surrounded by a variable amounts of neutrophils admixed with scattered macrophages. Pleura expanded by immature connective tissue composed of a mixture of proliferating fibroblast, new vessel formations and abundant macrophages, with occasional neutrophils; F) Hyalinization of the vessel wall and thrombosis in its lumen; G) Arteriole expanded with infiltrating neutrophils, macrophages and edema fluid.

The pleural surface was covered with a layer of fibrin with varying amounts of neutrophilic infiltrate in six cases (nos. 1–3 and 5–7). In addition to the fibrin tag, three rats (nos. 5–7) had increased pleural surface thickening with immature connective tissue composed of proliferating fibroblasts, neovascularization, abundant macrophages, and occasionally neutrophils. Six cases (nos. 1–3 and 5–7) had cardiac lesions that began in the epicardium and spread to the myocardium (Figure 3A-D). The atria were affected more severely than the ventricles. Furthermore, the severity of the lesions was greater on the right (5/6) than on the left side of the heart. The epicardium was markedly expanded by intense infiltrate of macrophages admixed with scattered neutrophils, lymphocytes, and mast cells. Cardiac myocytes are separated, surrounded, and lost in the subepicardial regions, with replacement by the inflammatory infiltrates described above.

Bacteriological finding

A pure culture of gram-positive coccobacilli was isolated from the affected organs. Based on biochemical properties, the organism was identified as *C. kutscheri*. In the PCR analysis, an isolate of 540 bp specific to *C. kutscheri* was obtained, which was consistent with this expectation (Figure 4). The non-spe-

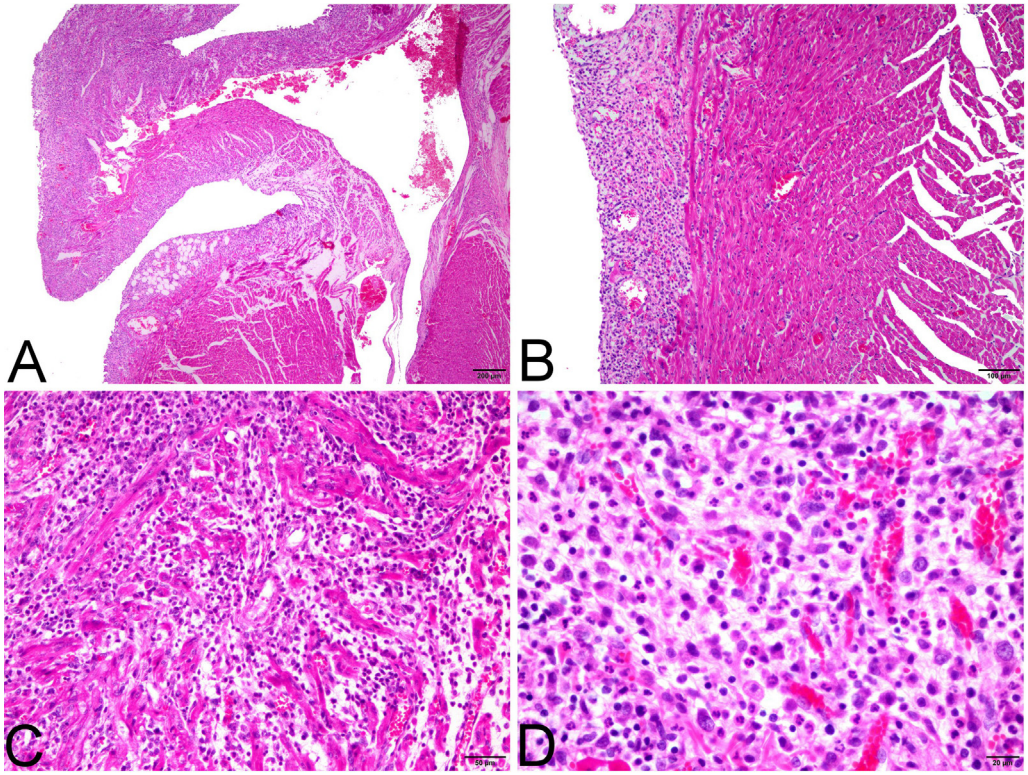

Figure 3. *Corynebacterium kutsheri*-associated cardiac lesions. H&E staining. A-B) Epicardium was markedly expanded by inflammatory infiltrates. These lesions spreading to the subepicardial myocardium. Note that the severity of atrial lesions is greater than that of the ventricle; C-D) Close-up images of the lesions. Cardiac myocytes are separated, surrounded, and lost with replacement by intense infiltrate of macrophages admixed with scattered neutrophils, lymphocytes and mast cells. Loss of cross striation in remaining cardiomyocytes.

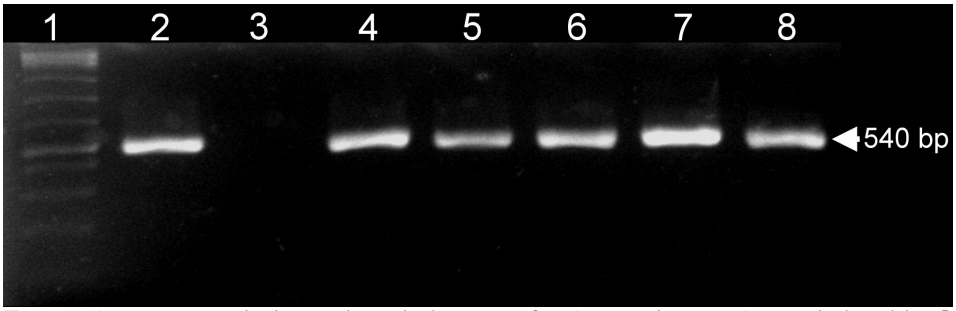

Figure 4. Agarose gel electrophoresis images of PCR products. PCR analysis with *C. kutsheri*-specific primers showing a 540 bp product as expected. Lane 1, 100-bp DNA ladder; Lane 1: positive control; Lane 2: negative control; Lane 3-7: tissue samples.

cific product was not determined. Based on the results of the bacteriological, biochemical, and PCR analyses,the causative agent of the disease was identified as *C. kutscheri*.

DISCUSSION

Corynebacteriosis, caused by *C. kutscheri*, was one of the first rodent diseases to be identified (Lemaistre & Tompsett, 1952). *C. kutscheri* has been found in the majority of rodent colonies studied in various countries (Otto et al., 2015). Despite reports that the measures taken have reduced the prevalence of infection, recent disease outbreaks indicate that *C. kutscheri* infection remains a threat to rodent colonies (Barthold, 2012; Won et al., 2007). In our literature review, we found no reports of *C. kutscheri* infection in rodent breeding units in our country. *C. kutscheri* infection is typically subclinical (Amao et al., 1995). As a result, it is critical to detect and exclude rodents infected with *C. kutscheri* prior to beginning experimental studies to obtain healthy results. Furthermore, outbreaks of clinical disease encountered during an experimental study, as in this study, may necessitate the study's termination, resulting in even greater losses (Saltzgaber-Muller & Stone, 1986). The clinical, macroscopic, histopathological, and bacteriological aspects of *C. kutscheri* infection, which emerged as a complication during an experimental study, were discussed in this study. This is the first study in our country to report *C. kutsheri* infection.

Infection with *C. kutscheri* in rodent colonies ranges from latent infections to fetal epizootic of pseudotuberculosis. Although tissue lesions are not asociated with latent infections, *C. kutscheri* can be isolated from many tissues of latently infected rodents (Barthold, 2012). Previous reports suggest that domestic animals become more susceptible to the diseases due to stress factors (Hazıroğlu et al., 2006; Tunca & Hazıroğlu, 2004; Tunca et al., 2006). *C. kutscheri associated* outbreaks are often caused by immune suppression under stressful conditions. Previous research has shown that immunosuppressive factors, such as malnutrition, transportation, overcrowding, high ammonia concentrations, and exogen administariton of immunosuppressive agents, such as cortisone and cyclophosphamide, can activate latent infections, leading to clinical disease (Shechmeister & Adler, 1953; Won et al., 2007; Zucker & Zucker, 1954). In this study, the rats that developed disease from *C. kutscheri* were not exposed to any of the stress factors listed above. It was interesting to see that the disease only appeared in rats exposed to swimming stress. There was no pathology found in the rats that were not swimming. Physical exercise, for example, activates adaptive responses, such as the sympathomedullary pathway and the hypothalamus–pituitary–adrenal axis, which release stress hormones to end the stress reaction and maintain homeostasis (Caplin et al., 2021). However, the excessive amount of stress hormones released during intense exercise may cause immunosuppression by suppressing bone marrow (Kruk et al., 2020). The emergence of disease only in the forced swim group in our study suggests that the stress induced by swimming contributed to the development of clinical disease by activating an existing latent infection under immunosuppression.

Clinical signs of *C. kutscheri* are often seen in the last phase of the disease (Barthold et al., 2012; Otto et al., 2015). Cachexia, hunched posture, periocular crusting, apathy to the environment, and respiratory distress reported in spontaneous or experimental *C. kutscheri* infection (Barthold et al., 2012; Giddens et al., 1968; Otto et al., 2015; Won et al., 2007) were also seen in this study. Porphyria and lameness reported by Otto et al. (2015) were not seen.

The location of lesions in *C. kutscheri* differs depending on the animal species; consistent with previous reports (Barthold et al., 2012; Giddens et al., 1968; Otto et al., 2015; Won et al., 2007), the majority of active infection was seen in rats' lungs. This study also found fibrinous pleuritis and mediastinal lymph node enlargement in addition to the pulmonary lesions seen in previous natural and experimental *C. kutscheri* infections (Giddens et al., 1968; Won et al., 2007). However, necrosuppurative hepatitis reported by Tuffery and Innes (1963) was not seen. It is unknown how the agent enters the lungs during active infection. The predominance of pulmonary lesions points to inhalation of contaminated aerosols as a possible mode of transmission. However, it is now widely accepted that the agent enters the lung via the hematogenous route (Barthold et al., 2012; Giddens et al., 1968; Otto et al., 2015). In our cases, lung lesions began at the interalveolar septum rather than the bronchioloalveolar border. This finding indicated that the agent entered the lungs through the hematogenous route rather than through inhalation. Furthermore, the random distribution of the pulmonary lesions in all lobes rather than just the cranial lobe (lesion limited to cranial lobe indicates spread by inhalation) supports the hematogenous route of transmission. As the disease progresses, diffuse involvement of the affected lung sections and pleuritis indicate that it spreads via the pericanalicular route. The lungs are affected in mild cases, but the airways are unaffected; however, severe lung lesions are accompanied by bronchial, bronchiolar, and tracheal lesions. This finding suggested that intracanalicular spread occurred later in the process. As a result, the agent was thought to have entered the lungs via the hematogenous route and spread via the pericanalicular and intracanalicular routes.

The first histopathological findings in *C. kutscheri* infection are foci of interstitial necrosis with neutrophil infiltration (Barthold et al., 2012; Otto et al., 2015). In line with this, we discovered that pulmonary lesions in rats infected with *C. kutscheri* began in the interstitium. Granulomatous foci in the lungs of *C. kutscheri*-infected rats were thought to be the first histopathological lesion in rats resistant to this infection (Giddens et al., 1968). However, neither a later study (Won et al., 2007) nor our findings support this hypothesis. It has been reported that initially observed interstitial lesions accompanied by neutrophil leukocytes enlarged and became surrounded by neutrophils and macrophages to form abscesses. In chronic cases, abscess foci are surrounded from the outside by a fibrous capsule (Barthold et al., 2012; Otto et al., 2015). Similarly, in *C. kutscheri*-infected rats, the most visible lung lesion was multifocal randomly distributed foci of colliquation necrosis with numerous viable and degenerated neutrophils. Neighboring lesions tended to merge into larger lesions. A demarcation area formed by neutrophils with karyorrhectic nuclei separated these lesions from the surrounding tissue. These lesions were surrounded by epithelioid histiocytes and giant cells in chronic

infection and only rarely by a fibrous capsule. Lung lesions were classified as pyogranulomatous lesions in this regard.

The pathological findings of *C. kutsheri* infections is non-specific (Barthold et al., 2012; Otto et al., 2015). Therefore, bacterial isolation via culture or PCR demonstration of *C. kutscheri*-specific gene(s) is important in the diagnosis (Fox et al., 1987; Jeong et al., 2013). However, the fact that C. kutscheri can be isolated from many tissues of latently infected rodents makes microbiological analysis challenging for disease diagnosis (Amano et al., 1991; Brownstein et al., 1985; Yokoiyama et al., 1975). Culturing the agents from the lesioned organs allows for a definitive diagnosis of the disease (Fox et al., 1987). The disease was diagnosed in the present study by isolating the agent from the lesioned tissues using bacteriological culture and demonstrating the genetic material of *C. kutscheri* in these lesions using the PCR technique. Moreover, using the Brown-Breen staining technique, a histochemical staining method, gram-positive bacteria were identified in the centers of the lesioned organs, allowing for a definitive diagnosis.

C. kutscheri has been isolated in various tissues and organs of subclinically infected rodents, including the oral cavity and submandibular lymph nodes (Amano et al., 1991; Brownstein et al., 1985). *C. kutscheri* has also been isolated up to day 90 from mice experimentally inoculated via the oronasal route (Yokoiyama et al., 1975). *C. kutschseri* infection, which was activated during an experimental application in rats and caused the development of pathological disease, was defined in this study. *C. kutscheri*'s zoonotic significance has grown in recent years. *C. kutscheri* has been isolated in cases of rat-bite fever caused by rodent bite, according to reports (Himsworth et al., 2014; Holmes & Korman, 2007). The present study revealed the disease threat faced by laboratory workers and animal caretakers in an experimental study due to the activation of *C. kutscheri* infection in rats subjected to swimming stress. Given the subclinical nature of *C. kutscheri* infections in rodent colonies, the study suggests that laboratory personnel should always take the necessary precautions when working with laboratory animals.

It has been reported that rodent species differ in their susceptibility to *C. kutsheri* infections. Intravenous administration of *C. kutscheri* to 15 different mouse breeds resulted in active infection in only seven of the breeds, with the remaining breeds remaining disease-free (Pierce et al., 1964). Although the exact cause of this difference between mouse breeds is unknown, Hirst and Campbell (1977) proposed that it is due to the mononuclear phagocytic system. They concluded that the monosystem phagocytic system effectively cleared the agent in resistant strains. Although there are studies presented in terms of susceptibility to the disease among mouse breeds, there is no study on which breeds are more susceptible in rat breeds. The Wistar breed rats were resistant by LeMaistre and Tompsett (1952). Infection occurs in these breeds only at high doses. Natural and experimental diseases have been observed in Sprague-Dawley rats in subsequent studies (Giddens et al., 1968; Won et al., 2007). The unavailability of reports of *C. kutscheri* infection in other breeds may be due to experimental studies' preference for Sprague-Dawley and Wistar breeds rather than disease resistance in these breeds. Natural *C. kutscheri* infection in Sprague rats was determined in the study under stress conditions caused by excessive exercise. However, active infection with *C. kutscheri* did not occur in all of the rats exposed to exercise stress. As a result, some of the rats exposed to stress developed the disease, whereas others did not, implying that, in addition to immunosuppression, individual differences play a role in disease development.

CONCLUSION

A natural *C. kutscheri* infection in Sprague-Dawley rats under stressful conditions induced by intense exercise was described in detail in terms of clinical, pathological, and microbiological perspectives in the present study. The subclinical course of *C. kutscheri* infections highlights the importance of routine *C. kutscheri* screening in experimental animal breeding facilities. *C. kutscheri*'s zoonotic significance suggests that staff working with experimental animals should pay special attention to this subject.

DECLARATIONS

Ethics Approval

All the experimental procedures were approved by the local ethics committee of Aydın Adnan Menderes University (approval no. 64583101/2021/091).

Conflict o Interest

The authors declare that no commercial funding was obtained that may be construed as potential conflict of interest.

Author contribution

Idea, concept and design: Eİ, ETE, RT

Data collection and analysis: Eİ, ETE, ÇN, RT

Drafting of the manuscript: Eİ, ETE, RT

Critical review: Eİ, RT

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Data Availability

The data are available from the corresponding author on reasonable request.

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NA.

REFERENCES

1. Amano, H., Akimoto, T., Takahashi, K. W., Nakagawa, M., & Saito, M. (1991). Isolation of Corynebacterium kutscheri from aged Syrian hamsters (Mesocricetus auratus). Laboratory Animal Science, 41(3), 265-268.

2. Amao, H., Akimoto, T., Komukai, Y., Sawada, T., Saito, M., & Takahashi, K. W. (2002). Detection of Corynebacterium kutscheri from the oral cavity of rats. Experimental Animals, 51(1), 99-102. https://doi.org/10.1538/expanim.51.99

3. Amao, H., Komukai, Y., Akimoto, T., Sugiyama, M., Takahashi, K. W., Sawada, T., & Saito, M. (1995). Natural and subc-

linical Corynebacterium kutscheri infection in rats. Laboratory Animal Science, 45(1), 11-14.

4. Amao, H., Moriguchi, N., Komukai, Y., Kawasumi, H., Takahashi, K., & Sawada, T. (2008). Detection of Corynebacterium kutscheri in the faeces of subclinically infected mice. Laboratory Animals, 42(3), 376-382. https://doi.org/10.1258/la.2007.06008e

5. Barthold, S. W. (2012). Corynebacterium kutscheri Infection, Liver, Mouse and Rat. In: T. C. Jones, J. A. Popp & U. Mohr (Eds.). Monographs on Pathology of Laboratory Animals: Digestive System (pp. 209-213). Springer Science & Business Media.

6. Brownstein, D. G., Barthold, S. W., Adams, R. L., Terwilliger, G. A., & Aftosmis, J. G. (1985). Experimental Corynebacterium kutscheri infection in rats: bacteriology and serology. Laboratory Animal Science, 35(2), 135-138.

7. Caplin, A., Chen, F. S., Beauchamp, M. R., & Puterman, E. (2021). The effects of exercise intensity on the cortisol response to a subsequent acute psychosocial stressor. Psychoneuroendocrinology, 131, 105336. https://doi.org/10.1016/j.psyneuen.2021.105336

8. Claudio, E. R., Endlich, P. W., Santos, R. L., Moysés, M.R., Bissoli, N. S., Gouvêa, S. A., Silva, J. F., Lemos, V. S., & Abreu, G. R. (2013). Effects of chronic swimming training and oestrogen therapy on coronary vascular reactivity and expression of antioxidant enzymes in ovariectomized rats. Plos One, 8(6), e64806. https://doi.org/10.1371/journal.pone.0064806

9. Fox, J. G., Niemi, S. M., Ackerman, J., & Murphy, J. C. (1987). Comparison of methods to diagnose an epizootic of Corynebacterium kutscheri pneumonia in rats. Laboratory Animal Science, 37(1), 72-75.

10. Giddens, W. E., Jr, Keahey, K. K., Carter, G. R., & Whitehair, C. K. (1968). Pneumonia in rats due to infection with Corynebacterium kutscheri. Pathologia Veterinaria, 5(3), 227-237. https://doi.org/10.1177/030098586800500304

11. Hazıroğlu, R., Şahal, M., Tunca, R., Güvenç, T., Duru, S. Y., & Ataseven, L. (2006). Pleuritis and pneumonia associated with nocardiosis and aspergillosis in a domestic short haired cat. Ankara Üniversitesi Veteriner Fakültesi Dergisi, 53(2), 149-151.

12. Himsworth, C. G., Zabek, E., Tang, P., Parsons, K. L., Koehn, M., Jardine, C. M., & Patrick, D. M. (2014). Bacteria isolated from conspecific bite wounds in Norway and black rats: implications for rat bite-associated infections in people. Vector Borne and Zoonotic Diseases, 14(2), 94-100. https://doi.org/10.1089/vbz.2013.1417

13. Hirst, R. G., & Campbell, R. (1977). Mechanisms of resistance to Corynebacterium kutscheri in mice. Infection and Immunity, 17(2), 319-324. https://doi.org/10.1128/iai.17.2.319-324.1977

14. Holmes, N. E., & Korman, T. M. (2007). Corynebacterium kutscheri infection of skin and soft tissue following rat bite. Journal of Clinical Microbiology, 45(10), 3468-3469. https://doi.org/10.1128/JCM.00607-07

15. Jeong, E. S., Lee, K. S., Heo, S. H., Seo, J. H., & Choi, Y. K. (2013). Rapid identification of Klebsiella pneumoniae, Corynebacterium kutscheri, and Streptococcus pneumoniae using triplex polymerase chain reaction in rodents. Experimental Animals, 62(1), 35-40. https://doi.org/10.1538/expanim.62.35

16. Kruk, J., Kotarska, K., & Aboul-Enein, B. H. (2020). Physical exercise and catecholamines response: benefits and health risk: possible mechanisms. Free Radical Research, 54(2-3), 105-125. https://doi.org/10.1080/10715762.2020.1726343

17. Lemaistre, C. & Tompsett, R. (1952). The emergence of pseudotuberculosis in rats given cortisone. The Journal of Experimental Medicine, 95(4), 393-408. https://doi.org/10.1084/jem.95.4.393

18. Otto, G. M., Franklin, C. L., & Clifford, C. B. (2015). Biology and diseases of rats. In: J. G. Fox, L. C. Anderson, G. M. Otto, K. R. Pritchett-Corning, & M. T. Whary (Eds.). Laboratory Animal Medicine (pp. 151-207). Academic Press.

19. Pierce-Chase, C. H., Fauve, R. M., & Dubos, R. (1964). Corynebacterial pseudotuberculosis in mice. I. Comparative susceptibility of mouse strains to experimental infection with Corynebacterium Kutscheri. The Journal of Experimental Medicine, 120(2), 267-281. https://doi.org/10.1084/jem.120.2.267

20. Ruehl-Fehlert, C., Kittel, B., Morawietz, G., Deslex, P., Keenan, C., Mahrt, C. R., Nolte, T., Robinson, M., Stuart, B. P., Deschl, U., RITA Group, & NACAD Group (2003). Revised guides for organ sampling and trimming in rats and mice--part 1. Experimental and Toxicologic Pathology, 55(2-3), 91-106.

21. Saltzgaber-Muller, J., & Stone, B. A. (1986). Detection of Corynebacterium kutscheri in animal tissues by DNA-DNA hybridization. Journal of Clinical Microbiology, 24(5), 759-763. https://doi.org/10.1128/jcm.24.5.759-763.1986

22. Shechmeister, I. L., & Adler, F. L. (1953). Activation of pseudotuberculosis in mice exposed to sublethal total body radiation. The Journal of Infectious Diseases, 92(3), 228-239. https://doi.org/10.1093/infdis/92.3.228

23. Tuffery, A. A., & Innes, J. R. M. (1963). Diseases of laboratory mice and rats. In: W. Lane-Petter (Ed.). Animals for research (pp. 47-108). Academic Press

24. Tunca, R. & Hazıroğlu, R. (2004). Kobaylarda deneysel Candida albicans enfeksiyonunda patolojik bulgular. Ankara Üniversitesi Veteriner Fakültesi Dergisi, 51(2), 133-142.

25. Tunca, R., Haziroglu, R., Guvenc, T., Kutsal, O., & Ozsoy, S. Y. (2006). Congenital cerebellar hypoplasia associated with BVD-MD virus infection in a naturally infected calf. Veterinarski Arhiv, 76(5), 453-460.

26. Won, Y. S., Jeong, E. S., Park, H. J., Lee, C. H., Nam,

K. H., Kim, H. C., Park, J. I., & Choi, Y. K. (2007). Upregulation of galectin-3 by Corynebacterium kutscheri infection in the rat lung. Experimental Animals, 56(2), 85-91. https://doi.org/10.1538/expanim.56.85

27. Yokoiyama, S., Ando, T., Hayano, K., & Fujiwara, K. (1975). Jikken dobutsu. Experimental Animals, 24(3), 103-110.

28. Zucker, T. F., & Zucker, L. M. (1954). Pantothenic acid deficiency and loss of natural resistance to a bacterial infection in the rat. Proceedings of the Society for Experimental Biology and Medicine, 85(3), 517-521.

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Case Report

A different treatment approach for Bovine papillomavirus in an Arabian horse

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ABSTRACT

A wart lesion was observed near the anus of a female Arabian horse housed in a private equine facility in Bucak District of Burdur Province. During the histopathological examination of the mass, equine sarcoid was revealed. Polymerase chain reaction (PCR) revealed the presence of bovine papillomavirus (BPV) type 10 and type 12 viral genomes in the tissue. It was observed that the combined treatment approach of PAPILEND™® cream + Zylexis™ + AlquermoldTM premix powder was beneficial in treating the sarcoid associated with BPV type 10 and type 12 on the anal region. No recurrence of the lesion was observed during the 6-month follow-up period. In conclusion, it is recommended to diagnose the presence of BPV in horses with warts and to investigate the efficacy of this combined treatment approach in larger populations.

INTRODUCTION

Bovine papillomavirus (BPV) types 1, 2 and 13 (BPV-1, -2, -13) are known to cause sarcoids in horses and potentially infect other species (Chambers et al., 2003). Although the routes of BPV transmission in the ungulate population are not fully known, possible routes include direct or indirect skin contact, contaminated materials, environmental conditions, airborne flies, and vertical transmission. In horses, BPV lesions are commonly found all around the body, particularly in the paragenital region, thorax-abdomen, and head regions (Torrontegui & Reid, 1994). While various treatment approaches for wart lesions caused by BPV have been extensively studied in ruminant animals, there are limited studies focusing on BPV-related warts in horses. Therefore, more research is needed in this area. In this study, the detection of the presence of BPV and the treatment of the tumor in an Arabian horse with an equine sarcoid case are reported using molecular methods, histopathological and immunohistochemical techniques.

CASE

In a private equestrian facility in Bucak District of Burdur Province, 3-year-old female Arabian horses, previously used for racing but later sold due to leg nerve injuries, developed wart lesions on the anal wall (Figure 1). After capturing and restraining the animals, a 5g sample of the wart lesion was collected under appropriate conditions. A portion of the sample was fixed in 10% formalin solution for histopathological and immunohistochemical examinations, while the remaining part was transported to the laboratory in a sterile container under cold chain conditions. This study was approved by the Local Ethics Committee for Animal Care of the Burdur Mehmet Akif Ersoy University (11/04/2023- decision number: E-93773921-770-264906).

The tumor mass was used for histopathological and immunohistochemical examination. The mass fixed in 10% formalin solution, processed routinely and embedded in paraffin. Sections of 5µm thickness were cut using a microtome and stained with Hematoxylin-eosin (HE) examined under a light microscope. For immunohistochemical examination, the sections were mounted on polylysine slides and stained using the streptavidin biotin peroxidase method. The anti-HPV antibody [BPV-1/1H8 + CAMVIR] (ab2417) was used for the detection of the papillomavirus. The Mouse and Rabbit Specific HRP/DAB (ABC) Detection IHC kit (ab64264) and DAB chromogen were used as secondary kits. After counterstaining with Harris Hematoxylin, the prepared slides were covered with a cover slip and examined under a microscope.

Extraction (Qiagen) was performed from the mass, and the sample was analyzed using the conventional polymerase chain reaction (PCR) method with BPV type-specific primers (BPV type 1-type 14).

For treatment, PAPILEND™® cream was applied once a day to the wart area for 10 days. Zylexis™ was administered

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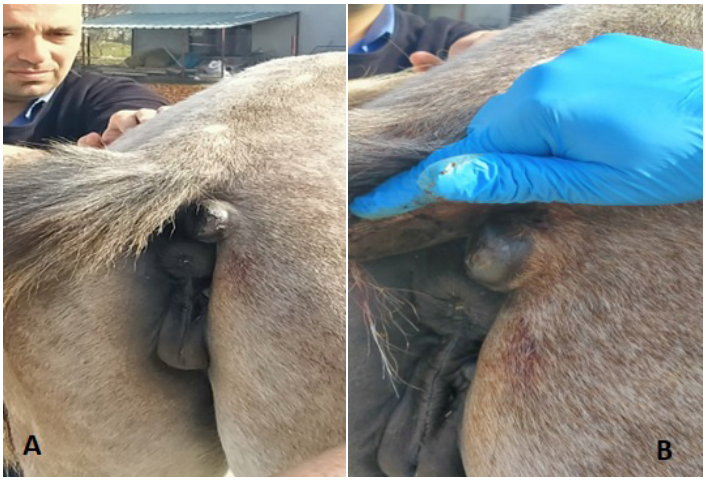

Figure 1. A) Gross appearance of the tumor localized near the anus of an Arabian horse.
B) Close-up view.

intramuscularly at a dose of 2 ml on days 0, 3, and 9 to activate the immune system. Additionally, 150 grams of Alquermold™ premix powder was added to the animal's daily feed for 10 days.

The histopathological examination of the mass revealed a prominent dermal proliferation of spindle-shaped fibroblasts arranged in a fascicular or interlacing pattern. The epidermis showed widespread hyperplasia, hyperkeratosis, and rete peg formation. Notably, fibroblasts at the dermo-epidermal junction exhibited a perpendicular orientation towards the basement membrane. Ulceration and inflammatory cell infiltration were observed on the surface of the mass. Increased vascularity and hyperemic vessels were also noted. In the immunohistochemical examination, positive reactions with papillomavirus were detected in some epithelial cells in the epidermis (Figure 2).

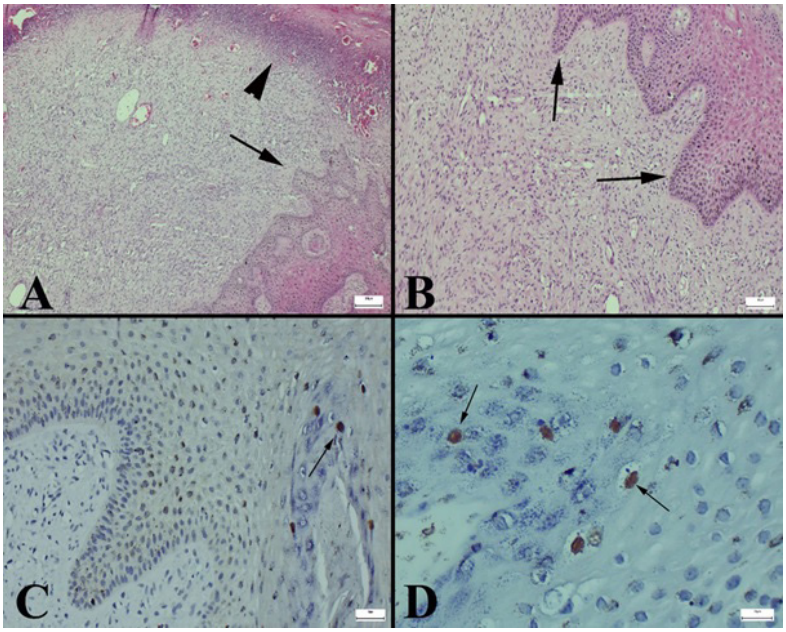

Figure 2. A) Histopathological appearance of the fibromatous type equine sarcoid, showing ulcer and inflammatory reaction on top of the mass (arrowhead) and rete peg (arrow). HE, Scale bar = 200µm. B) Higher magnification of rete pegs (arrows). HE, Scale bar = 100µm. C) Positive reaction for papilloma virus (arrows), Streptavidin Biotin Peroxidase method. Scale bar = 50µm. D) Higher magnification of papilloma virus positive epidermal cells (arrows),Streptavidin Biotin Peroxidase method. Scale bar = 100µm.

The PCR method using BPV type-specific primers (BPV type 1-type 14) applied to the tissue sample confirmed the presence of viral genomes belonging to BPV type 10 (Figure

3) and type 12 (Figure 4). The results were negative for the other types.

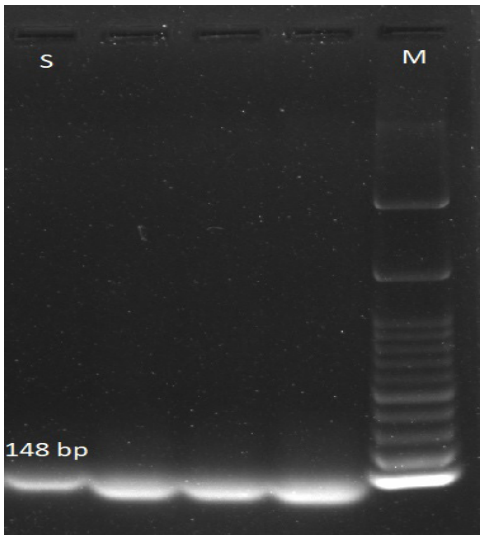

Figure 3. Detection of BPV type 10 by PCR.

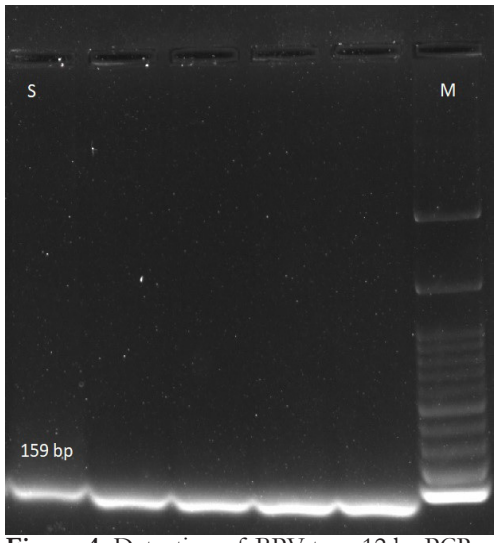

Figure 4. Detection of BPV type 12 by PCR.

As a result of the combined treatment with PAPILEND™® cream + Zylexis™ + Alquermold™ premix powder, regression and healing of the wart area in the anal region were observed (Figure 5). No recurrence of the lesion was observed during the 6-month follow-up period.

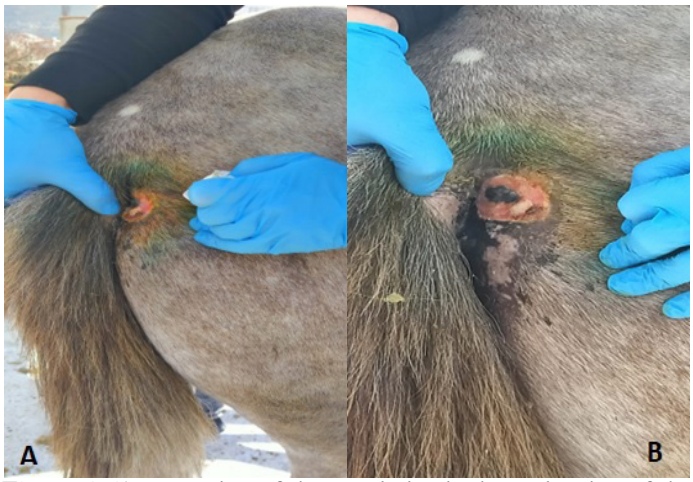

Figure 5. A) Regression of the wart lesion in the anal region of the Arabian horse.
B) Close-up view

DISCUSSION

Sarcoids, which are thought to be caused by BPV types of the Deltapapillomavirus genus commonly in horses, are characterized by localized lesions with intense fibroblastic proliferation and epidermal hyperplasia/dysplasia (Nasir & Brandt, 2013). In this case, for the first time, BPV types 10 and 12, which belong to the Xipapillomavirus genus, were detected in a wart that developed in the anal region of a horse. BPV types 10 and 12 genes found in the mass that have generally been detected in cattle mammary, body, and oral lesions in previous studies (Sökel and Kale, 2022; Özmen and Kale, 2023). In this study, a sarcoid case detected in a horse, which was previously used in races but sold due to leg nerve injury, and kept for recreation in a private riding facility located in an area nearby dairy cattle, is presented. The grazing areas of the horse and the cattle were intertwined, and close contact between the animals was observed. Experimental studies have shown the development of sarcoid-like lesions in horses following inoculation with BPV, while another study reported no formation of warts (Reid et al., 1994). Therefore, definitive statements cannot be made regarding the transmission of BPV from one horse to another or from cattle to horses. However, BPV DNA has been detected in horses' sarcoid mass and it has been suggested that flies (*Musca autumnalis*) carrying BPV DNA can act as vectors for BPV transmission (Kemp-Symonds, 2000). BPV generally spreads through direct or indirect contact, entering the skin through abrasions and lesions (Özmen and Kale, 2023). Therefore, in the case we examined, we suspect that inter-species transmission of BPV occurred through direct contact or via flies. Histopathological examinations of

equine sarcoids have identified fibroblastic and nodular types as the most common ones (Gebre et al., 2018; Kareem & Salman, 2019). Kareem & Salman (2019) determined that sarcoid prevalence is highest in males between the ages of 3-7. In this case, a fibroblastic-type sarcoid was diagnosed through histopathological examination of the wart in a 3-year-old female riding horse. BPV antigens were detected immunohistochemically in the epidermal cells. It has been found that sarcoids in horses occur most frequently in the head-neck region (51%), trunk and genital region (32.3%), and leg and shoulder areas (16.1%) (Gebre et al., 2018). The localized development of tumors in horses can lead to functional disorders depending on the affected area. As a result, animals may experience issues such as weakness, sensitivity, bleeding, blindness, difficulty in defecation and urination, locomotion problems, difficulties in parturition, feeding, and chewing (Gebre et al., 2018). Currently, there is no standardized method for the treatment of equine sarcoids. However, various treatment methods such as surgical procedures (conventional excision and CO_2 laser excision), cryotherapy, hyperthermia, radiotherapy, chemotherapy, immunotherapy, topical immunomodulation, and antiviral agents are used (Taylor & Haldorson, 2013). The topical cream (PAPILEND™®) used in this case was developed to soften wart-like formations seen in cattle and reduce their adverse effects by inducing hardening. The cream's composition includes glacial acetic acid, salicylic acid, garlic oil, tea tree oil, glyceryl monostearate, stearic acid, cetyl stearyl alcohol, hydrogenated castor oil, podophyllum, and water. It has been reported that some of the ingredients in the cream cause warts to regress and disappear due to their topical cytotoxic and antimitotic effects (Rivera & Tyring, 2004;). BPV is not a strong immunogen in mammalian organisms and does not induce significant inflammation, except for local cellular immunity. Therefore, the main objectives in combating the virus include the development of neutralizing antibodies, stimulation of cellular immunity, elimination of infected cells producing early proteins, and exposing keratinocytes to the virus (Araibi et al., 2004). To stimulate the immune system, an immunostimulant called Zylexis™ was used. Vitamin E, selenium, copper, and zinc are important substances for animals in strengthening the immune system against diseases and promoting keratin production (Sökel and Kale, 2022). Therefore, Alquermold™ premix powder was used. The primary goal of topical treatments in equine sarcoid cases is to stimulate a local immune response to eliminate tumor cells.

CONCLUSIONS

We recommend conducting investigations on BPV cases to explore the sequence and phylogeny, as new types and variants of the virus continue to emerge. In this regard, we suggest diagnosing the presence of BPV in a larger population of animals with equine sarcoid cases and subsequently monitoring the outcomes of this combined treatment approach.

DECLARATIONS

Ethics Approval

This study was approved by the Local Ethics Committee for Animal Care of the Burdur Mehmet Akif Ersoy University (11/04/2023- decision number: E-93773921-770-264906).

Conflict of Interest

The authors declare that they have no conflict of interests.

Author Contribution

Idea, concept, and design: Y.S., M.K.

Data collection and analysis: Y.S., M.K., O.O.

Drafting of the manuscript: Y.S., M.K.

Critical review: S.H., Y.Y., K.A.

Data Availability

The data used to prepare this manuscript are available from the corresponding author when requested.

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REFERENCES

1. Araibi, E. H., Marchetti, B., Ashrafi, G. H., & Campo, M. S. (2004). Down regulation of major histocompatibility complex class I in bovine papillomas. Journal of General Virology, 85, 2809–2814. https:// doi: 10.1099/vir.0.80128-0.

2. Chambers, G., Ellsmore, V. A., O'Brien, P. M. Reid, S. W. J., Love, S., Campo, M. S. & Nasir, L. (2003). Association of bovine papillomavirus with the equine sarcoid. Journal of General Virology, 84, 1055–1062. https:// doi: 10.1099/vir.0.18947-0.

3. Gebre, D., Gizachewu, A., & Negero, J. (2018). Equine sarcoid: Epidemiology and its clinical impact in working donkeys in selected districts of Central Ethiopia. Journal of Veterinary Science and Technology, 9(537), 2. https:// 10.4172/2157-7579.1000537.

4. Kareem, M. H., & Salman, S. S. (2019). Types and histopathological characteristics of equine sarcoid. Online Journal of Veterinary Research, 23(2), 112-120.

5. Kemp-Symonds, J. G. (2000). The Detection and sequencing of Bovine Papillomavirus type 1 and 2 DNA from *Musca autumnalis* (Diptera: Muscidae) face flies infesting sarcoid-affected horses. MSc Thesis. London, UK: Royal Veterinary College.

6. Nasir, L., & Brandt, S. (2013). Papillomavirus associated diseases of the horse. Veterinary Microbiology, 167, 159–167. https:// doi: 10.1016/j.vetmic.2013.08.003.

7. Özmen, G., & Kale, M. (2023). Searching bovine papillomavirus presence in lesions seen on teats of cows. Pesquisa Veterinária Brasileira, 43, e07150. https://doi.org/10.1590/1678-5150-PVB-7150.

8. Reid, S. W., Gettinby, G., Fowler, J.N., & Ikin, P. (1994). Epidemiological observations on sarcoids in a population of donkeys (*Equus asinus*). Veterinary Record, 134, 207–11. https://doi: 10.1136/vr.134.9.207.

9. Rivera, A., & Tyring, S. K. (2004). Therapy of cutaneous human papillomavirus infections. Dermatologic Therapy, 17(6), 441-448. https://doi: 10.1111/j.1396-0296.2004.04047.x.

10. Sökel, S., & Kale, M. (2022). Sığırların vücutlarında görülen bovine papillomavirus kaynaklı siğillerin virolojik, Patolojik ve klinik açıdan incelenmesi. PhD Thesis, Burdur, Turkey. Burdur Mehmet Akif Ersoy Üniversitesi, Sağlık Bilimleri Enstitüsü.

11. Taylor, S., & Haldorson, G. (2013). A review of equine sarcoid. Equine Veterinary Education, 25(4), 210-216. https://doi:10.1111/j.2042-3292.2012.00411.x.

12. Torrontegui, B. O., & Reid, S. J. (1994). Clinical and pathological epidemiology of the equine sarcoid in a referral population. Equine Veterinary Education, 6, 85–88. https://doi.org/10.1111/j.2042-3292.1994.tb01098.x